STUDY OF HIGH SENSITIVE C-REACTIVE PROTEIN AND MYOGLOBIN IN THE SALIVA OF ACUTE MYOCARDIAL INFARCT PATIENTS

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In partial fulfillment for the Degree of

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BRANCH VI

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CERTIFICATE

This is to certify that this dissertation titled **"STUDY OF HIGH SENSITIVE C-REACTIVE PROTEIN AND MYOGLOBIN IN THE SALIVA OF ACUTE MYOCARDIAL INFARCT PATIENTS"** is a bonafide dissertation performed by **P.SHANMUGAPRIYA** under our guidance during the post graduate period 2010 – 2013.

This dissertation is submitted to **THE TAMILNADU DR. M. G. R. MEDICAL UNIVERSITY,** in partial fulfillment for the degree of **MASTER OF DENTAL SURGERY in ORAL PATHOLOGY AND MICROBIOLOGY, BRANCH VI.** It has not been submitted (partial or full) for the award of any other degree or diploma.

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CONTENTS

ABSTRACT

Background: Salivary diagnostics is an emerging field that has progressed through several important developments in the past decade. Identification and standardization of salivary biomarkers can be used to develop compact point-of-care devices for rapid analysis and this can be used as an accurate screening tool for the diagnoses of systemic diseases in the near future.

Objective: To evaluate the presence of high sensitive C-reactive protein (hs-CRP), Myoglobin(MYO) in the unstimulated whole saliva (UWS) of acute myocardial infarct patients and controls and thereby investigate the utility of saliva as an alternative diagnostic fluid for Acute Myocardial Infarction (AMI).

Materials and Methods: UWS was collected from AMI patients at three different time periods; 6-12 hours, 24-48 hrs and 48-72 hrs of onset of signs and symptoms of AMI and the salivary hs-CRP and myoglobin expression was compared with normal controls. Hs-CRP was evaluated using Immunoturbidimetric assay (IT) and myoglobin using Enzyme linked immunosorbant assay (ELISA).

Results: Expression of salivary hs-CRP levels (mg/L) were higher in AMI group than normal controls. Salivary myoglobin levels showed increase in expression in periodontitis controls and normal controls when compared to the AMI group samples collected at 24-48 hrs and 48-72 hrs (P=0.013 and P= 0.022 respectively). In AMI patients myoglobin levels showed a significant increase in samples collected at 6-12 hrs (P=0.018) than in the samples collected at 24-48 hrs and 48-72 hrs (P=0.009 and P=0.015 respectively). The salivary myoglobin expression showed a significant increase in the samples collected at 6-12hrs from NSTEMI patients (P=0.043) than in the samples collected from STEMI patients in AMI group.

Conclusion: Hs-CRP using Immunoturbidimetry showed consistent results, while myoglobin results by ELISA were inconsistent, this inconsistency we feel was due to its sensitivity to long term storage. It is thus preferable to analyze the samples immediately or prevent repeated freeze-thaw cycle. Salivary analysis of cardiac biomarkers needs more sensitive techniques of high sensitivity and specificity to be utilised as a diagnostic tool and further studies are to be done to arrive at a reference range for salivary hs-CRP and myoglobin levels.

Keywords: Unstimulated whole saliva, salivary biomarkers, hs-CRP, Myoglobin, Acute Myocardial Infarction

Introduction

Saliva as a diagnostic tool has the potential to revolutionize screening technology for systemic as well as malignant diseases. Identification and standardization of salivary biomarkers are being done to develop compact point-of-care devices for rapid analysis that can be used as an accurate screening tool for the diagnosis of systemic diseases[1].

Human saliva is a biological fluid with enormous diagnostic potential. Because saliva can be non-invasively collected, it provides an attractive alternative for blood, serum or plasma. It has been postulated that the blood concentrations of many components are reflected in saliva. Saliva harbours a wide array of proteins, which can be informative for the detection of diseases. Profiling the proteins in saliva over the course of disease progression could reveal potential biomarkers indicative of different stages of diseases, which may be useful in medical diagnostics[2]. Like blood, saliva contains many protein and RNA molecules, both of which are encoded by genes[3].

Whole saliva or Oral fluid is composed primarily of saliva secreted by the three major paired salivary glands the parotid, submandibular and sublingual and numerous minor salivary glands located throughout the oral cavity, it also contains gingival crevicular fluid, sloughed oral epithelial cells, nasopharyngeal discharge, food debris, and bacteria and their products, despite this, whole saliva reflects the secretions of major salivary glands and hence a valuable tool in research and diagnostic purposes[4, 5, 6] .

Early diagnosis of myocardial infarction (MI) is a clinical challenge and there are numerous evolving algorithms. Salivary biomarkers including C-reactive protein, myoglobin and myeloperoxidase were identified as potential diagnostic markers along

with electrocardiogram findings and serum biomarkers in acute myocardial infarct (AMI) patients[7].

Currently, AMI is identified by detection of rise and/or fall of cardiac biomarkers (preferably cardiac troponin-I/cTnI) together with evidence of myocardial ischemia based on ECG or imaging[8]. Despite tremendous progress in developing new diagnostic and screening methods, a substantial number of AMI cases today are missed or diagnosed too late to offer effective therapies. There is a subset of patients in whom there is no ECG changes (Non-ST elevated MI) at the initial stage, in these patient's serum biomarkers are the only tool to diagnose myocardial necrosis. However these measurements require time and delays therapy and affect prognosis[7].

C-reactive protein (CRP) is an acute phase reactant that is produced by the liver in response to stimuli from inflammatory cytokines. It is non-specific but a sensitive marker of systemic inflammation. Conventionally CRP is measured as a systemic marker in tissue injury and infection, and therefore conventional CRP assays are calibrated to measure dynamic increase in CRP concentration and not able to detect low levels of increase due to subtle inflammation as in atherosclerosis[9].

The newer high sensitive CRP (hs-CRP) assays measures low level increase of CRP[9, 10]. Measurement of elevated CRP by high sensitivity assays serves as a predictor or risk factor for atherosclerosis progression in apparently healthy individuals and also serves as a prognostic factor in patients with already diagnosed atherosclerotic disease[11].

Myoglobin (MYO), the oxygen binding heme protein constitutes about 2% in both skeletal and cardiac muscle. Myoglobin is the first marker to rise after AMI. The advantages of myoglobin in early diagnosis of myocardial infarction are its high early

sensitivity and the possibility of rapidly assessing the success of thrombolytic therapy[12].

In this study our objective was to identify and assess salivary biomarkers which are seen in serum of patients with myocardial infarction. In this study we are going to evaluate the levels of hs-CRP and MYO in the saliva of AMI patients. These biomarkers are increased in the serum of AMI patients[7, 12, 13].

Aims and Objectives

AIM

To evaluate the presence of high sensitive C-reactive protein (hs-CRP) and Myoglobin (MYO) in the unstimulated whole saliva (UWS) of acute myocardial infarct (AMI) patients and controls.

OBJECTIVES

- To evaluate the presence of high sensitive C-reactive protein (hs-CRP) in the UWS of AMI patients using Immunoturbidimetric assay (IT).
- To evaluate the presence of myoglobin (MYO) in the UWS of AMI patients using Solid phase Sandwich- Enzyme linked immunosorbant assay (ELISA).
- To evaluate the presence of hs-CRP, and MYO in the UWS of controls.

NULL HYPOTHESIS

Levels of hs-CRP and MYO are same in the saliva and serum of AMI patients.

Materials and Methods

INSTITUTIONAL REVIEW BOARD APPROVAL

Institutional Review Board (IRB) approval was obtained for this project (Annexure I A & I B). The study protocol was submitted to the IRB (Annexure II). All samples were collected after obtaining informed consent from the subjects. (Annexure III)

PATIENT SELECTION AND SAMPLE COLLECTION

To evaluate 30 study subjects (AMI patients) and 30 controls (healthy subjects) which include 15 with periodontitis and 15 without periodontitis of both gender in the adult population.

The Study subjects were AMI patients who were admitted with the signs and symptoms of AMI to the cardiac care unit (CCU) of Madras Medical Mission hospital (MMM), Madurai Meenakshi Mission Hospital and Research Centre (MMHRC) and Voluntary health services and hospital (VHS).

Study controls were patients attending the outpatient department of Ragas Dental College and Hospitals.

STUDY GROUPS (Annexure IV)

Group I: Unstimulated saliva from 30 acute myocardial infarct patients

Group II: Normal subjects (controls)

- IIA - Unstimulated saliva from 15 controls with periodontitis
- IIB - Unstimulated saliva from 15 apparently healthy controls without periodontitis

Inclusion criteria for Group I

Subjects with signs and symptoms of MI[8].

- Ischemic chest pain
- Subjects showing ST elevation in ECG
- Subjects without ST elevation but with clinical symptoms of MI.

Exclusion criteria for Group I

Subjects who had fever, stroke and autoimmune diseases were excluded

Inclusion criteria for Group II A

Subjects with periodontitis

Criteria used for assessing Periodontitis[14]:

1. Plaque scores more than 30% of sites
2. Bleeding scores more than 30% of sites
3. Probing depth greater than or equal to 3mm
4. Clinical attachment loss greater than or equal to 2mm

Inclusion criteria for Group II B

Healthy, age and gender matched subjects.

Exclusion criteria for Group II B

- Subjects without cardio vascular disease and other metabolic and systemic diseases such as Diabetes mellitus.
- Subjects who were not on antibiotics or any other medications during the past 2 weeks

METHOD FOR COLLECTING SALIVA (Group I)

- Unstimulated saliva from AMI patients was collected thrice, first sample was collected within 6-12 hours of onset of signs and symptoms of AMI, second sample after 24 hours and before 48 hours and third sample after 48 hours and before 72 hours of onset of sign and symptoms.
- Unstimulated whole saliva was collected in a sterile container.

Saliva collection in group I

1. Patients were conscious and were able to breathe without the help of oxygen mask.
2. Patients were seated in a semi reclined comfortable position
3. The patients were given drinking water (bottled) and asked to rinse their mouth out well (without drinking the water).
4. Five minutes after this oral rinse, the patients were asked to spit whole saliva (WS) into a sterile container.
5. The subjects were instructed to refrain from talking. The subject was asked to drop down the head and let the saliva run naturally to the front of the mouth, hold for a while and spit into the sterilized container provided for about once a minute for up to 10 minutes. The goal for each whole saliva donation was approximately 5ml. The subjects were asked not to cough up mucus or vomit. (Samples diluted with mucus or vomit was discarded).
6. Collected samples were labelled with a sample id number and kept at 4 degree Celsius during transport.
7. Detailed case history and all medications taken and prescribed were documented.
8. Time and date of sample collection was recorded

Things to avoid: (Prior to collection of second and third samples)

1. Brushing teeth within 1 hour prior to collection.

2. Using salivary stimulants: chewing gum, lemon drops, granulated sugar.

3. Consuming a major meal within 1 hour prior to collection.

4. Consuming acidic or high sugar foods within 20 minutes prior to collection.

Method for collecting Saliva (Group II A and II B)

Salivary collection was done according to the technique by Navazesh *et al* [15]. Unstimulated whole saliva was collected from controls between 9 to 12 am. The subjects were advised to rinse his or her mouth several times with drinking water. The subjects were instructed to refrain from talking. The subjects were asked to drop down the head and let the saliva run naturally to the front of the mouth, hold for a while and spit into the sterilized container provided, about once a minute for up to 10 minutes. The goal for each whole saliva donation was approximately 5ml.

Things to avoid:

1. Brushing teeth within 1 hour prior to collection.

2. Using salivary stimulants: chewing gum, lemon drops, granulated sugar.

3. Consuming a major meal within 1 hour prior to collection.

4. Consuming acidic or high sugar foods within 20 minutes prior to collection.

5. Consumption of alcohol 12 hours prior to collection of saliva.

STORAGE

- Collected samples were centrifuged at 3500rpm for 15 to 20 minutes at -20 degrees Celsius.

- The supernatant was aliquoted in 4 separate micro centrifuge tubes and sediments were stored separately. Samples were stored at -70 degrees Celsius till analysis in the deep freezer of Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospitals.

ARMAMENTARIUM

1. Oral examination devices (Gloves, Mask, Mouth mirror, Williams probe)
2. Centrifuge Tubes (1.5ml)
3. Micropipettes
4. Micropipette tips (20µl, 100µl, 200 µl,1000 µl)
5. Cooling Centrifuge
6. -70 degrees Celsius ultra low temperature storage cabinet
7. Sterile containers for saliva collection
8. Ice pack (for transfer)
9. MYOGLOBIN ELISA kit (Biocheck inc^{TM}.USA)

   Automatic washer

   ELISA reader
10. HsCRP Immunoturbidimetric kit (BioSystems S.A^{TM}, Spain)

    CS400 automatic analyser

Myoglobin levels were assessed by ELISA and the absorbance measured at 450 nanometres (nm) and hs-CRP levels were assessed by IT assay and the absorbance measured at 450 nm.

PROCEDURE

Enzyme linked Immunosorbant Assay (ELISA-Biocheck.inc™.USA) for quantitative determination of Myoglobin.

Contents and Reagents

Antibody-Coated Wells (1 plate, 96 wells)

Microtiter wells coated with murine monoclonal anti-myoglobin.

Reference Standard Set (1.0 ml/vial, 1 set/kit)

Prediluted (10 fold) standards contains 0, 25, 100, 250, 500, and 1000 ng/ml myoglobin, liquid, ready-to-use.

Enzyme Conjugate Reagent (22 ml/vial)

Contains goat anti-myoglobin antibody conjugated to horseradish peroxidise in Tris Buffer-BSA solution with preservatives.

Substrate-TMB Reagent (11 ml/bottle)

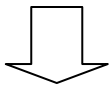
Contains one-step TMB solution.

Stop Solution (1 bottle, 11 ml/bottle)

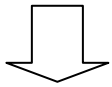
Contains diluted hydrochloric acid (1N HCl).

ASSAY PROCEDURE - ELISA

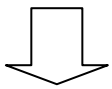
All reagents were brought to room temperature (18-25°C) before use.



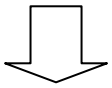
To the 96 antibody coated microtitre wells, 20µl of prediluted myoglobin standards (std≠1 to std≠5), controls and undiluted whole saliva samples were added to the appropriate wells.



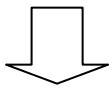
200 µl of Enzyme Conjugate Reagent was added into each well, and incubated at room temperature for 45 minutes.



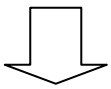
The incubated microwells were rinsed 5times with distilled water by automatic washer and the residual water drops removed by striking the wells onto absorbent paper.



100 µl of TMB Reagent solution was added into each well and incubated at room temperature for 20 minutes



As the standards and samples develop blue colour, the reaction was stopped by adding 100 µl of stop solution into each well.



After all the blue colour changes into yellow colour completely, the microtitre wells were read at an absorbance of 450 nm by a microtitre well reader.

Quality control-ELISA

- The absorbance (Y axis) of the colour complex is then measured and the generated values for each pre diluted standard were plotted against the expected concentration (X axis) forming a standard curve. This standard curve was then used to accurately determine the concentration of myoglobin in the samples tested. (Annexure V)

IMMUNOTURBIDIMETRIC ASSAY (IT) TO DETERMINE hs-CRP (BioSystems S.A^{TM}. Spain)

Contents and composition

1. Reagent A(1x40 ml): Glycine buffer 0.1 mol/L, sodium azide 0.95 g/L
2. Reagent B (1x10 ml): Suspension of latex particles coated with anti-human CRP antibodies, sodium azide 0.95g/L.
3. Hs-CRP Standard

Assay procedure

- The working reagent was prepared by pouring Reagent B vial into Reagent A bottle.
- The working reagent, the standard and the sample were brought to room temperature (37°c).
- 1.5 ml of working reagent and 20 µl of the standard and sample is pipetted into the cuvette and the cuvette is inserted into the instrument (CS400 auto analyser)
- The absorbance is measured at 450nm after 10 minutes.

Quality control-IT

The absorbance of each point of the standard curve was calculated (Y axis) and the values were plotted against the hs-CRP concentration (X axis). The hs-CRP concentrations in the samples were calculated by interpolation of its absorbance in the calibration curve. (Annexure V)

STATISTICAL ANALYSIS

The **sample size** of 30 in each group has been determined from the Standard Statistical Table (Expecting an effect value of 0.30% to detect the minimum difference between control and study groups under an approximate power of 80% with the level of significance of 0.05).

Data entry, database management and all statistical calculations performed with the aid of the Statistical Package for the Social Sciences ^{TM} (SPSS, version 17) software. **Descriptive statistics** were calculated for all variables.

Differences in means between more than two groups were assessed using the **analysis of variance** (ANOVA), followed by Tukey's Honestly significant difference **(Tukey HSD)** test.

To compare the expression of salivary myoglobin and hs-CRP between group I and group II **t-test** was done. A P value of **< 0.05** was considered to be statistically significant.

Review of Literature

Saliva is an aqueous glandular fluid with a low protein content[16] secreted into the mouth by three pairs of major salivary glands: Parotid, submandibular and sublingual glands and numerous minor salivary glands present in the labial mucosa, buccal mucosa, soft palate and tongue. The salivary secretion is either mucous i.e. rich in mucoproteins or serous i.e. watery or mixed both serous and mucous[17].

Physiology of saliva

Saliva possesses several important functions involving the oral health and maintaining homeostasis. It protects the oral mucosa by lubrication, helps in speech, perception of taste and preliminary breakdown of food particles and also acts as a defence against microorganisms and helps in the maintenance of enamel mineralization as well as inhibits demineralization[18].

Healthy adults produce approximately 500-1500ml of saliva per day at about 0.5ml/min. There are lot of physiologic and pathologic conditions that affect the production of saliva both in quality and quantity like smell, taste, circadian rhythm, mental and physical state, hormones, drugs, age, oral hygiene etc[19].

Salivary secretion and composition is controlled by autonomic nervous system, by both sympathetic and parasympathetic branches innervating the salivary glands[4]. The minor salivary glands produce saliva continuously and the factors affecting the secretion of major salivary glands have little effect on minor glands. The parotid secretion is the major source of stimulated salivary secretion particularly acid-stimulated saliva has a composition more closer to parotid secretion. The major composition of unstimulated whole saliva is from submandibular glands[17].

Composition of saliva

The composition of saliva collected varies depending upon the particular mixture of secretions from the different glands through different type of stimulation. Salivary output and composition depend on the activity of the autonomic nervous system: the sympathetic system controls the serous part of the gland and the mucous part influenced by both sympathetic and parasympathetic system. The difference in stimuli and receptor action can modify the quantity, viscosity and ionic and protein concentrations of saliva secreted. The presence of food in the mouth can also affect salivary composition[19]. The terms whole saliva and/ or oral fluid denotes the fluid component of mouth containing the secretions from the major and minor salivary glands plus the gingival crevicular fluid and oral mucosa collected without stimulation by passive drooling or spitting[17] .

Salivary composition varies in relation to the serous or mucous component of the glands[20] ; the relative contribution of each type of gland to total unstimulated saliva secretion varies from 65%, 23%, 8% to 4% for submandibular, parotid, von Ebner and sublingual glands respectively[15] .

The term oral fluid and / or unstimulated whole saliva (UWS) denotes that the components of saliva also have a non glandular origin, Oral fluid is composed primarily of saliva secreted by the three major paired glands—parotid, submandibular, and sublingual—and numerous minor glands located throughout the oral cavity. Whole saliva also contains gingival crevicular fluid, sloughed oral epithelial cells, nasopharyngeal discharge, food debris, and bacteria and their products[4] .

A compositional analysis of oral fluid may be important for its implications in physiology, pathology and diagnosis of oral and systemic diseases. Saliva is composed

of Inorganic, Organic, Non-protein, Protein/Polypeptide, Hormone and Lipid molecules[19, 21]

Inorganic components

Whole saliva is composed mainly of water and electrolytes[19] ($Na^{+,}$ $K^{+,}$ Mg^{2+}, Ca^{2+}, Cl^{-}, HCO_3^{-}, HPO_3^{2-}).

Organic components

This includes non-protein and lipid molecules. Eg: Uric acid, bilirubin and creatinine are detected in saliva. Saliva also contains glucose, amino acids, lipids like cholesterol and mono/diglycerides of fatty acids[19]. The content of free fatty acids and partial glycerides was high in saliva compared to that in blood and lymph, because of absence of lipase activity[21].

Proteins and Polypeptides in Saliva

Saliva contains large number of proteins. Proteins derived from salivary gland productions are amylase, proline rich proteins (PRPS), cystatins, carbonic anhydrase, secretory Ig-A and mucins[19,4]. Proteins are also from plasma leakage through gingival crevicular fluid like albumin, Ig-G, transferrin[19] . Salivary proteins have wide range of functions, such as protection of oral tissues, defence against microorganisms, host immune response and in digestion. Other functions of salivary proteins are in remineralization of enamel, in taste perception and also in cell signalling activities[19] Other proteins that are present in saliva are enzymes and enzyme inhibitors, hormones such as growth factors, and cytokines such as interleukin-8[22] .

Saliva as a diagnostic tool

Interest in saliva as a diagnostic medium has increased in the past decade. Saliva, gingival crevicular fluid, and mucosal transudates are being used for drug monitoring and for the detection of various oral and systemic maladies since 1982[23]. Oral fluid or whole saliva (WS) is a complex biological fluid. WS has got three contributors:

1. Exocrine portion by the major and minor salivary glands with a protein concentration ranging between 150 to 400 mg %, exocrine contributions from salivary glands are synthesized locally by specialized cells and secreted upon sympathetic and parasympathetic stimulation so contribute to difference in salivary flow rates and differences in the concentrations of salivary constituents.
2. Non exocrine contributors can be either host-derived components such as desquamated epithelial cells, intact and remnants of blood cells, gingival fluid, and possibly fluid entering the oral cavity through mucosal seepage.
3. Non host-derived components are microorganisms and food residues. Non exocrine WS constituents are also derived from local sites and/or are synthesized elsewhere in the body. For example, gingival fluid is a serum transudate rich in protein, and enters the oral cavity through the gingival crevice, it reflects the protein component in serum. Oral cavity is the portal of entry to the gastrointestinal tract and also communicates with the posterior nasal passage and upper respiratory tract so the whole saliva can have components from these sites, and such components could point toward a

variety of systemic diseases[4]. Thus saliva can be a useful diagnostic tool in local as well as systemic diseases.

Technological developments have influenced the advances in the salivary diagnosis, a wide range of molecular markers in the saliva are studied and compared to serum levels including microbes, chemicals and immunological markers[23] . Increasing interest in saliva based diagnosis and analysis is because saliva is a biological fluid and like blood, saliva contains many protein and RNA molecules, both of which are encoded by genes[24]. Blood and its components are the mainstay for laboratory diagnostic procedures, other biological fluids like urine and cerebrospinal fluid are also used for diagnosis of disease, thus saliva can also be advantageous in certain situations[1]. Saliva, is easy to collect, does not clot so handling is easy, low-cost storage and is safe for the operator and the patient[19, 24]

Saliva is a biologic fluid source that provides many, if not most, of the same molecules found in the systemic circulation. This makes saliva a potentially valuable fluid for the diagnosis of various systemic diseases[1]. Salivary diagnosis also has an advantage of use as point-of-care device which can be used at home or in a resource limited setting.

Transfer of molecules from blood to saliva

The processes by which plasma compounds can enter saliva are[19,25]

1. Ultrafiltration: This is through gap junctions between cells of secretory units (acinar and ductal cells) of salivary glands. Molecules which are lesser than 1900 Da can enter saliva like this and their concentration in saliva is 300 – 3000 times lower than in plasma.

2. Transudation: Entry of plasma compounds into oral cavity, from crevicular fluid or from oral mucosa eg: albumin
3. Selective transport of lipophilic molecules through passive diffusion.
4. Active transport through protein channels, by ligand receptor binding.

Advantage of using saliva as diagnostic tool

Whereas blood permeates all tissues and therefore can contain molecular constituents arising from all organs of the body, other body fluids are more restricted in their association with select organs. Thus a major advantage of using other body fluids is that the diagnostic tests will be more specific for the biomarkers of the associated tissue unlike blood where all organs can shed their molecules. Saliva also has the advantage of being a sample that can be collected by non-invasive means and it also contains plasma components which enter it through active transport and passive diffusion. Saliva as a diagnostic medium has been closely associated with aero-digestive system and biomolecules of systemic diseases have also been identified including those for endocrine function, stress or psychological state, exposure to infectious agents, use or metabolism of drugs or other xenobiotics, and cancers[4,26].

Salivary diagnostics has entered a new era with identification of human salivary proteome which is important for determining oral health and disease pathogenesis[27, 28] and with infusion of federal funds (US) to integrate nanotechnologies and microfluidic engineering concepts into developing compact point-of-care devices for rapid analysis of this secretion[1]. As of November 2007 more than 1000 proteins have been identified in human saliva[24].

Saliva, a scientific and clinical entity familiar to every oral health researcher and dental practitioner, has emerged as a translational and clinical commodity that has

reached national visibility in the United States of America at the National Institutes of Health and the President's Office of Science and Technology. "Detecting dozens of diseases in a sample of saliva" was issued by President Obama as one of the 14 Grand Challenges for biomedical research in the 21st Century (National Economic Council, 2010)[29]. The clinical utility of saliva as a diagnostic medium depends upon the type of saliva being analysed either glandular or whole saliva. Unstimulated whole saliva is used in majority of diagnostic purposes[1].

Salivary diagnosis in Dentistry

Salivary analyses have been used in dentistry and oral diseases, to estimate the risk of appearance, diagnosis of disease, monitoring of disease progression and evaluation of therapy efficacy for caries, periodontitis, premalignant and malignant oral lesions, as well as infectious diseases of the oral cavity, can be assessed by analysing different constituents of saliva. Individuals at risk of caries can be identified using tests that determine saliva flow rate, saliva buffer capacity, and colonisation of the oral cavity by cariogenic bacteria[26].

Saliva as a diagnostic aid has got its use in periodontal disease diagnosis, prognosis and monitoring therapeutic response. Salivary analysis has been carried out to identify proteins, immunological markers, host cells, inflammatory mediators, hormones, bacteria and bacterial products[30].

Saliva as a diagnostic tool in systemic diseases

Early detection of disease is important in managing a disease and reducing the morbidity and mortality by preventing and/or delaying the complications. To achieve this goal medical research is directed towards identification of molecular biomarkers

which can help in identifying a disease at the earlier stages before complications develop[31].

A biomarker or biologic marker, according to the working group definition[32] "is a substance that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"**.** These molecular biomarkers could be nucleic acids or proteins which can reflect the individuals physiological status and abnormality in these markers present in body fluids can be used for disease detection[31]. Most systemic diseases such as cancer, cardiovascular, metabolic, and neurological diseases are very challenging to diagnose without supplementary clinical evaluation[33]. Limitations to realise early disease detection are[33]

1. Lack of definitive molecular biomarkers for specific diseases

2. Lack of an easy and inexpensive sampling method with minimal discomfort

3. Lack of an accurate, easy-to-use, and portable platform to facilitate early disease detection

The discovery of salivary biomarkers and the ongoing development of salivary diagnostic technologies is the solution to overcome these limitations. Saliva as a diagnostic tool is used in research and diagnostic studies of systemic diseases involving salivary glands and oral cavity like Sjögren syndrome.

Sjögren's syndrome (SS) is a systemic autoimmune disease with a variety of presenting symptoms which may delay its diagnosis. Sixteen WS proteins were found to be down-regulated and 25 WS proteins were found to be up-regulated in primary SS patients compared with matched healthy control subjects. These proteins reflected the

damage of glandular cells and inflammation of the oral cavity system in patients with primary SS[34]. Three protein biomarkers, cathepsin D, alpha-enolase and beta-2-microglobulin (B2M), and three mRNA biomarkers, myeloid cell nuclear differentiation antigen (MNDA),Guanylate binding protein 2 (GIP2) and low affinity IIIb receptor for the Fc fragment of IgG (FCGR3B), were significantly elevated in patients with pSS compared to both SLE patients and healthy controls[35]. In both the above mentioned studies paraffin- stimulated WS and glandular saliva specimens from individual major salivary glands is collected[34, 35]. The technique used to identify proteins and mRNA biomarkers in WS samples is Mass spectrometry, expression microarray profiling [34], western blotting or ELISA and quantitative polymerase chain reaction(qPCR)[35].

In a study to identify the significant proteins in saliva in primary sjogren syndrome using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry(SELDI- TOF- MS) and two-dimensional difference gel electrophoresis(2D-DIGE) identified significant increases of b-2-microglobulin, lactoferrin, immunoglobulin (Ig) j light chain, polymeric Ig receptor, lysozyme C and cystatin C in all stages of SS. Two presumed proline-rich proteins, amylase and carbonic anhydrase VI, were reduced in the patient group[36].

Saliva is used as a diagnostic tool in Viral and bacterial diseases

An oral fluid based test for detecting HIV antibodies which has got accuracy equivalent to serum test and much safer and easier to use has been introduced as early as 1997 in the United states[37, 38]. A study to analyse the utility of saliva as a diagnostic medium for the detection of antibodies to human immunodeficiency virus type 1 (HIV-1) and HIV-2 under nonlaboratory conditions using a self-contained kit (Saliva z

Strip [ST]), which combines the collection and processing, as well as the analysis, of the specimen. The result showed good performance for HIV -2 positive sera[39]. The available immunoassay kits to analyse HIV antibodies in sera were found to be effective to detect HIV antibodies in Oral fluid[40, 41].

Anti HCV antibodies were assayed in saliva of patients undergoing haemodialysis using Immunoassay[42].

Epidemiological investigation of a Hepatitis A outbreak which included salivary antibody testing revealed new Hepatitis A cases as well as showed anti HAV antibody 10 days after passive immunization[43]. Oral fluid samples were compared with serum samples as a specimen source for hepatitis A, B, and C virus markers. Oral fluid was obtained with a treated absorbent pad and tested by using existing commercial enzyme immunoassays with only minor modifications. Compared with serum sampling the sensitivity and specificity of oral sampling were 100% (51 of 51 samples) and 98% (46 of 47 samples) for hepatitis A virus immunoglobulin M, 100% (29 of 29 samples) and 100% (29 of 29 samples) for hepatitis B virus surface antigen, and 100% (13 of 13 samples) and 100% (13 of 13 samples) for hepatitis C virus antibody, respectively. The decline of hepatitis A virus immunoglobulin M in oral samples was parallel to, though somewhat more rapid than, that of hepatitis A virus immunoglobulin M in serum samples[44].

A study using an antigen capture anti-DENV IgA (ACA) ELISA technique, utilising saliva from dengue confirmed patients detected dengue virus (DENV)–specific immunoglobulin A (Ig A) early in the phase of a dengue infection, the sensitivity is better for(100%) secondary infection, than primary infection(36%)[45].

Helicobacter pylori was detected in saliva of patients with upper gastro intestinal symptoms using PCR[46,47]. The presence of antibodies to other infectious organisms such as Borrelia burdogferi, Shigella or Tenia Solium can also be detected through the saliva[48].

Salivary diagnostics in metabolic diseases

A study by Radhika S and Ranganathan K[49] on oral candidal carriage and oral salivary glucose levels in Type 2 diabetes, aimed to observe that if salivary glucose levels monitoring can be used as a non invasive diagnostic tool for glycemic control. Both stimulated and unstimulated saliva was collected from Type 2 diabetes subjects and normal healthy controls, glucose levels determined by glucose- oxidase method, showed comparable saliva-plasma levels, and salivary glucose levels were significantly higher in diabetics than in non-diabetics.

The discovery of salivary mRNA biomarkers using micro array analysis and verified by reverse transcription quantitative real time PCR has resulted in validating candidate mRNA biomarkers of which 2 were upregulated and 2 were down regulated in type 2 diabetes mellitus (T2DM) and can be used for early detection of T2DM[50].

Salivary levels of Ig E, nerve growth factor, myoglobin and insulin is observed to be elevated in T2DM subjects and individuals with autoimmune disorders when compared to controls[51].

Salivary biomarkers in Malignancies

Salivary soluble fragments of HER2/neu[52] and metabolite analysis of saliva samples of 215 individuals with oral, pancreatic and breast cancers (n=69,18 and 30 respectively) and 11 periodontal disease patients and 87 healthy controls identified a total of 57 metabolites using capillary electrophoresis time-of-flight mass spectrometry

(CE-TOF-MS). The resultant profiles manifested relatively higher concentrations of most of the metabolites detected in all three cancers in comparison with those in people with periodontal disease and control subjects. This suggests that cancer-specific signatures are embedded in saliva metabolites[53].

Candidate protein biomarkers (3 proteins) in the saliva of lung cancer patients from 72 saliva samples of lung cancer patients and healthy controls were identified using Two- Dimensional Difference Gel Electrophoresis (2D-DIGE) and Mass spectrometry(MS) technique and these biomarkers were further validated by immunoassay[54].

Salivary transcriptomic and proteomic analysis of 10 breast cancer pations and 10 matched controls using micro array and 2D-DIGE and comparison with prevalidated (previously discovered transcriptomic and proteomic biomarkers in breast cancer patients) samples of a different cohort revealed that there are significant variations in salivary molecular biomarkers between breast cancer patients and matched controls. Eight mRNA biomarkers and one protein biomarker, which were not affected by the confounding factors were identified[55].

Study to identify candidate protein biomarkers for early detection of oral squamous cell carcinoma (OSCC) from human salivary proteome resulted in identification of five candidate biomarkers (M2BP, MRP14, CD59, catalase, and profilin) detected independently in OSCC patients compared with healthy controls[56].

Salivary biomarkers in cardiovascular diseases

A group of salivary biomarkers including C-reactive protein, myoglobin and myeloperoxidase were identified as potential diagnostic markers along with electrocardiogram findings in acute myocardial infarct patients[7]

A study utilizing unstimulated WS and comparing with serum level of Acute Myocardial Infarct (AMI) Patients showed significant increase of creatinine phosphokinase levels in the first and second day of AMI, in both saliva and serum when compared to healthy controls, this can be useful in rapid diagnosis of AMI and can reduce the mortality and morbidity of myocardial infarct patients[57]. The cardiac specific isoform of creatinine kinase, creatinine kinase MB (CK MB) is found to be elevated in both unstimulated WS and serum of-care testing of acute MI[58].

Salivary lysozyme levels were found to be significantly increased in patients with oral infection, hyperglycemia and hypertension and can be a early stage biomarker for cardiovascular disease detection[59,60].

Salivary biomarkers in stress

The National Institute for Occupational Safety and Health USA (NIOSH) uses salivary IgA as a noninvasive immunologic biomarker of occupational stress[61]. An assessment of salivary cortisol, salivary Ig A(sIgA) and chromogranin A(CgA) among dental students before a academic test showed significant increase of salivary cortisol levels[62]. These biomarkers are also found to be increased in individuals with occupational stress[63].

Measuring of salivary alpha-amylase (sAA) and salivary cortisol (sC) appear to be feasible to investigate direct stress effects in relation to naturalistic traffic noise exposure in a laboratory setup[64].

Salivary biomarkers in other diseases

Salivary Total Cholestrol(TC), Triglycerides(TG), Low Density Lipoprotein-Cholestrol(LDL-C) and Very Low Density Lipoprotein-Cholestrol(VLDL-C) concentrations were significantly high in patients with ischemic stroke when compared with risk and control groups. So based on this study it can be concluded that lipid profile can be assessed in saliva and can be used as simple, monitoring tool in stroke-prone individuals with reasonable accuracy[65].

Oral fluid is used for testing the drug level concentration[66, 16]. Oral fluid, is used as an alternative specimen with detection time of drugs and metabolites relatively short and similar to blood, many drugs have analyte concentrations in the oral fluid that are generally proportional to those found in blood[16].

Levels of Salivary C-reactive protein, an inflammatory marker is compared in patients with Hashimoto's thyroiditis (HT) and sub acute thyroiditis (SAT). SAT patients had significantly elevated salivary CRP levels compared to HT patients and controls. Thus saliva CRP levels can be used to serve as inflammatory markers in SAT patients and may aid their clinical evaluation[67].

Methods of saliva collection

Oral fluid (whole saliva) is for the larger part produced and secreted by the various salivary glands, such as the submandibular, sublingual, and parotid glands[4]. Each type of salivary gland secretes a characteristic type of saliva[19]. For example, sublingual saliva is very rich in mucins and contains little amylase, while parotid saliva, which is the main source of salivary amylase and basic proline-rich proteins, does not contain mucin[4]. The composition of saliva varies, depending upon whether salivary secretion is basal or stimulated.

The submandibular glands are the major contributors to resting (unstimulated) saliva, and the parotid glands are the major contributors to stimulated saliva. The contribution of sublingual glands to unstimulated and stimulated whole saliva is low[15].

Saliva is being studied extensively and is being used for risk assessment, diagnosis and monitoring high-risk behavior and disease progression. The major disadvantage in the use of saliva for health-related purposes is the lack of standardization in saliva collection methods[15].

Several physiological and pathological conditions influence the collection of saliva of particular importance in clinical research and diagnostic research is the time of the day and duration of collection. For reliable monitoring of the functional potency of a salivary gland, it is recommended to collect both unstimulated and stimulated saliva during a period of 10 minutes for diagnostic research purposes. For further standardization, patients must be instructed to refrain from eating and drinking at least 90 min prior to the test session, and to avoid swallowing and oral movements during collection. To minimise the effects of diurnal variation, it is advised to collect all saliva samples at the same, fixed time of the day[4,15].

Techniques for collecting glandular specific saliva

Glandular saliva specimens from individual parotid glands were collected into Lashley cups (placed over the orifices of the stenson's duct) and from the submandibular/sublingual glands by syringe aspiration (from the orifices of the Wharton's duct, located anteriorly in the floor of the mouth), respectively[34].

Techniques for collecting unstimulated whole saliva

The patient is advised to refrain from intake of any food or beverage (water exempted) one hour before the test session. Smoking, chewing gum and intake of

coffee also are prohibited during this hour. The subject is advised to rinse his or her mouth several times with deionized (distilled) water and then to relax for five minutes[15].

The unstimulated WS can be collected by[25]

1. Draining / passive drooling method.
2. The spitting method
3. The swabbing method

Techniques for collecting stimulated whole saliva

Here the salivary flow is stimulated by asking the patient to chew on a piece of paraffin and or by chewing on a sugar candy or by applying citric acid on the tongue[15,25].

There are companies that manufacture commercial saliva collection devices for diagnostic and research purposes. These include: Salimetrics oral swabs (http://www.salimetrics.com); Oasis Diagnostics® VerOFy® I/II; DNA_SAL™ (http://www.4saliva.com); OraSure Technologies OraSure HIV specimen collection device (http://www.orasure.com); CoZart® drugs of abuse collection devices (http://www.concateno.com); and the Greiner Bio-One Saliva Collection System (http://www.gbo.com)[26].

Saliva storage

Saliva specimens, after collection, is aliquoted and frozen immediately to maintain the integrity of the sample, from the time of collection saliva should be kept in ice till final analysis. The refrigeration not only prevents degradation of saliva molecules but also prevents contamination by bacterial growth. Saliva contains

bacterial protease enzymes which can degrade several salivary proteins, this can affect protein compound investigation[19].

Collection of saliva into ice cooled vials is recommended to slow down the activity of hydrolytic enzymes present in saliva. Further, collection in vials containing a cocktail of protease inhibitors, including ethylene diamine tetra acetic acid **(**EDTA), phenyl-methane-sulfonyl-fluoride **(**PMSF), soy bean trypsin inhibitor, and E-64, is in particular useful when saliva is collected for protein purification purposes to prevent proteolysis. Bacterial and cellular debris are directly removed after collection by centrifugation[4]. The method of specimen storage influences the concentration of the compounds to be analysed, as some salivary analytes have a very short half life and undergo rapid disintegration (catecholamine) and some can stay stable at room temperature for upto a week(Ig G)[19]

A general approach to avoid degradation of salivary compounds[19,68]:

1. Immediately store saliva aliquots without any processing. Specimens can often be stored at room temperature (when analysis is carried out immediately or in 30–90 min from collection), at +4 °C (when analysis is carried out in 3–6 h from collection), at −20 °C and better at −80 °C (when analysis is carried out days to months after collection).

2. Snap-freezing of saliva in liquid nitrogen: mix each saliva aliquots with an equal volume of 80% glycerol in H2O, then dip the sample in liquid nitrogen. This storage procedure aims to inhibit the bacterial protease activity degrading some salivary protein compounds, such as s-IgA .

3. Inhibition of the enzyme activity present in saliva: mix each saliva aliquot with enzyme inhibitors 10:1 (leupeptin, aprotinin and 4-[2-aminoethyl] benzenesulfonyl fluoride)

4. Addition of sodium azide (NaN3) to saliva specimens in attempt to retard bacterial growth. The use of sodium azide does not influence the measurement of salivary markers when serum-based immunoradioassays are modified for saliva, not even if these methods involve separation or extraction steps. But the possible interference of sodium azide with horseradish peroxydase, a common component of enzyme immunoassays, must be taken into account.

General considerations and limitations in saliva storage and processing[4]

The method of collecting, storing and processing saliva should be ideal for all types of salivary biomarkers. The specimen collection and storage should be determined by the type of diagnostic research and by the stability of the salivary biomarker. The freezing/ thawing and prolonged storage at low temperatures affect the pH and viscoelasticity of the saliva and the presence of bacterial hydrolytic enzymes in saliva should also be taken into consideration.

The advantage of using saliva as diagnostic tool is because of its non-invasive nature, and feasibility where blood sampling is not possible and its ease of use as a tool in resource limited settings, but saliva collection, handling and storage has its own limitations as it has to be centrifuged, refrigerated and/ frozen immediately to prevent degradation of the biomarkers.

Salivary analysis

Analysis of saliva is done either to study a group of compounds or to determine a single compound in oral fluid. Most often kits available for serum or plasma assay is modified to analyse saliva[19].

Salivary ionic concentrations are determined by ion selective electrodes, atomic absorption and spectrometric methods[19].

Salivary proteins structure and function are studied with lots of techniques like, liquid chromatography, two dimensional gel electrophoresis, capillary electrophoresis, mass spectrometry (MALDI-TOF), nuclear magnetic resonance, immunoassays and proteomic analysis of saliva[53,54,36,27].

The barriers to widespread implementation of saliva diagnostics derive from technological problems in achieving sensitivity, miniaturization, high throughput, automation, portability, low cost, high functionality, and speed to enable high-content chemical and biochemical analyses. Microfluidic systems can be designed to obtain and process measurements from small volumes of complex fluids, such as saliva, with efficiency and speed, and without the need for an expert operator. The National Institute of Dental and Craniofacial Research (NIDCR-USA) has initiated a concerted research effort in the area of saliva diagnostics. The NIDCR held a workshop on "Development of New Technologies for Saliva and Other Oral Fluid-Based Diagnostics" in September1999. In 2002 NIDCR funded a series grants to develop technologies to measure and analyze multiple substances in saliva at a great speed[4].

Salivary diagnostics has entered a new era with identification of human salivary proteome which is important for determining oral health and disease pathogenesis[27,28] and with infusion of federal funds(US) to integrate nanotechnologies

and microfluidic engineering concepts into developing compact point-of-care devices for rapid analysis of salivary secretion[1]. As of November 2007 more than 1000 proteins have been identified in human saliva[24]

The NIDCR funded device developing programs are[4,33]:

- A parallel diffusion immunoassay optical based system this is capable of imaging several surface-linked immunoassays simultaneously. The aim of this system is to measure small molecule analytes such as hormones and drugs in whole saliva.
- An integrated platform that includes electrochemical sensors for oral cancer detection.
- Microfluidics platform, for detection of either a cytokine profile or oral bacteria, is based on a microchip electrophoretic immunoassay (μCEI) that relies on photolithographically fabricated molecular sieving gels to enrich the sample and a laser-induced fluorescence detector system
- Integrated microfluidic platform for the detection of multiple pathogens based on an on-chip polymerase chain reaction/reverse transcriptase-polymerase chain reaction (PCR/RT-PCR) system.

In addition to these microfluidics projects, the NIDCR funded salivary diagnostic technology development also supported development of a two bead –based sensor arrays. This bead-based microchip platform with fluorescence and optical detection capabilities has been adapted for a broad range of analyte classes including pH, electrolytes, metal cations, sugars, biological cofactors, toxins, proteins, antibodies, and oligonucleotides. The initial focus by using this is kept on cardiac risk assessment.

The second bead array is based on optical fibres and its initial work focuses on asthma and renal patients. The seventh project focuses on the validation of a first-generation oral chip for the identification and quantification of oral microbes by using high through put DNA-micro array. These systems allow miniaturization, integration, and multiplexing of complex functions, enabling such systems to move closer to the patient.

The objective is to develop an integrated microfluidics platform where sample collection, processing and analyses of cellular as well as soluble analytes can be done. Advances in diagnostic technology promise inexpensive devices for performing real-time analysis of a largenumber of proteins, nucleic acids, small molecules, and drugs in oral fluids, in the comfort of their own homes[4].

Cardiac biomarkers use in Acute Myocardial infaction

Ischemic heart disease (IHD) is the leading cause of death worldwide for both men and women (7 million totals per year). IHD is the generic designation for a group of pathophysiologically related syndromes resulting from *myocardial ischemia*—an imbalance between the supply (perfusion) and demand of the heart for oxygenated blood [69]. In more than 90% of cases, the cause of myocardial ischemia is reduced blood flow due to obstructive atherosclerotic lesions in the coronary arteries. Thus, IHD is often termed coronary artery disease (CAD) or coronary heart disease.

IHD usually presents as one or more of the following clinical syndromes:

- *Myocardial infarction*, the most important form of IHD, in which ischemia causes the death of heart muscle.
- *Angina pectoris*, in which the ischemia is of insufficient severity to cause infarction,

but may be a harbinger of MI.

- *Chronic IHD with heart failure*.
- *Sudden cardiac death* [69.]

Epidemiology

IHD in its various forms is the leading cause of death for both males and females in the United States and other industrialized nations. Each year nearly 500,000 Americans die of IHD[69]. but, the identification of major risk factors through population-based studies and effective control strategies combining community education and targeted management of high risk individuals have contributed to the fall in CVD mortality rates (inclusive of coronary and stroke deaths) that has been observed in developed countries. At present the developing countries contribute a greater share to the global burden of CVD, 5.3 million deaths attributable to CVD occurred in the developed countries in 1990, whereas the corresponding figure for the developing countries ranged between 8 to 9 million (ie, a relative excess of 70%)[70.]

The burden of cardiovascular disease is rising in India. The estimated prevalence of coronary heart disease is around 3–4% in rural areas and 8–10% in urban areas among adults older than 20 years, representing a twofold rise in rural areas and a six-fold rise in urban areas over the past four decades[71].

There are no web-based disease registries for Cardio Vascular Diseases in India[72]. Several cross sectional studies in India shows a rising trend in coronary artery disease in urban India[70]. It has been estimated that India had the highest number of deaths (over 1.5 million) in the world due to CAD in 2002, which is expected to double from 1985 to 2015[72]. In India the average age for first acute myocardial infarction is 53 years[73]

The INTERHEART study[74] done as a case control study in 52 countries observed that younger individuals are affected more with coronary heart disease, attributing the risk to non-conventional and genetic factors. But in the south Asian population and especially India there is a increase in morbidity and mortality due to CHD associated with increase in conventional risk factors like diabetes and smoking, another important observation is that there is an increase in risk factors even among controls less than 60 years of age. Further, the long-term case fatality following acute coronary syndrome is considerably higher among Indians as compared to other populations[75] this combined with lack of support mechanisms for evidence based treatment and follow up for AMI, results in almost 49.1 per cent higher mortality among the poor as compared to richest.

Findings from the South Asian component of INTERHEART study convey important messages to the health care providers and policy makers in the country as all these conventional risk factors for CHD are potentially modifiable and are good starting points for prevention [76] based on this study *ii)* early detection of persons with risk factors and cost-effective interventions for reducing risk; and *(iii)* early detection of persons with clinical disease and cost-effective secondary prevention measures to prevent complications is to be the aim of health care providers, this also implies the importance of identification of new markers and development of newer and cost effective technologies to screen the population at large for risk of CVDs especially in resource constrained situations.

Salivary biomarkers in Cardiovascular diseases

In this study our objective is to detect cardiac biomarkers in the saliva of patients admitted with signs and symptoms of acute myocardial infarction and we have selected a panel of biomarkers based on studies conducted in western countries[7,77, 78].

The National Institute of Dental and Craniofacial Research (NIDCR-USA) is funding a program-"Development and validation technologies for saliva based diagnostics" from 2002 the aim of this program is to use nanotechnology to develop point of care devices and in 2006 the fund was extended for four programmes and one of this is headed by Dr. John McDevitt (Rice University, Houston, TX) who is developing salivary diagnostic device for detection of cardiac biomarkers for acute myocardial infarction[6]

Currently the criteria for diagnosis of myocardial infarction is made if one of the following criteria is present[8]:

1. Detection of rise and /or fall of cardiac biomarkers (preferably troponin)with at least one value above the 99th percentile of the upper reference limit together with evidence of myocardial ischemia with at least one of the following:
    - Symptoms of ischemia
    - ECG changes indicative of ischemia [new ST-T changes or new left bundle branch block (LBBB)]
    - Development of pathological Q waves in the ECG
    - Imaging evidence of new loss of viable myocardium or new regional wall motion abnormality.

Based on these criteria, a patient is diagnosed as having a ST-elevated myocardial infarction (STEMI), non-ST- elevated myocardial infarction (NSTEMI) or

unstable angina. The serum biochemical markers of myocardial infarction include Myoglobin (MYO), Creatinine kinase-MB (CK-MB), total CK, and cardiac troponin T (c-TnT) and cardiac troponin I (c-TnI)[79] .The rate and time of release of these serum biomarkers are dependent upon various factors and the delay in sample procurement, processing and analysis may be inadequate for early diagnosis and effective intervention. Use of saliva for diagnosis of AMI is being explored because of the potential possible early diagnosis, possibly in the ambulance, home or in the clinic[7] . Studies with standard immunoassays showed that cardiac enzymes are present in saliva and levels of these enzymes were found to be elevated in patients with AMI[1,7].

Inflammatory markers of cardiovascular disease

Cardiovascular disease is multifactorial and chronic inflammation is one of the basic mechanism for atherosclerosis, rupture of the atherosclerotic plaque and the subsequent thrombi being the main cause for AMI, so inflammatory markers such as c-reactive protein[80,81] and Myeloperoxidase[82] are used as additional risk factors in determining acute cardiac events as well as to predict future risks and also for screening susceptible population[83,84].

C-Reactive protein (CRP)-Structure,Synthesis and clinical charecteristics

CRP is a phylogenetically highly conserved plasma protein found both in vertebrates and invertebrates. CRP was first discovered by Oswald Avery as acute phase reactant in patients infected with *Streptococcus pneumoniae,* sera obtained from these patients in the acute phase of illness were found to have a protein that can precipitate the “C” polysaccharide in the pneumococcal cell wall[85].

CRP is a 118 KD ubiquitous protein belonging to the pentraxin family of proteins and is a calcium-binding protein[77]. CRP has got a lot of biological activities, it

can activate the classical complement pathway, stimulate phagocytosis and can bind to immunoglobulin receptors[85]. In humans, CRP is produced by the liver and its production and release regulated by cytokines such as Tumor necrosis factor-α (TNF-α), Interleukin 1β(IL-1β) and IL-6[77]. However extra hepatic sources of human CRP have been suggested in several studies and the sources include neuronal cells in Alzheimer's disease, smooth muscle cells, macrophages in atherosclerotic plaques and adipose tissues[86], and smooth muscle cells of diseased coronary venous bypass grafts[87] but these extra hepatic sources doesn't increase the serum levels of CRP significantly, they are thought to be a part of the local environmental inflammatory response[85]

CRP is produced by everyone average value in an adult is 1.5 mg/L, the levels are high in individuals who smoke, have hypertension, in obese individuals and levels tend to be lower in thin, athletic individuals; however CRP levels in an individual also have a genetic influence. CRP is increased in infectious conditions in acute stage of the infection and can be used to predict the course of infection, CRP is increased in chronic inflammatory diseases like Rheumatoid arthritis and is tested to monitor therapeutic efficiency and progress of disease[80].

CRP and Cardiovascular disease

The traditional major risk factors for cardiovascular diseases are cigarette smoking, diabetes mellitus, hyperlipidemia and hypertension, but the absence of these factors does not exclude the development of cardiovascular disease. Data from studies by Ridker PM and Rifai N *et al*[88] suggest that the C-reactive protein level is a stronger predictor of cardiovascular events than the LDL cholesterol level and that it adds prognostic information to that conveyed by the Framingham risk score. It has been found that patients with lower low density cholesterol one of the risk factors for

cardiovascular disease developed CVDs and this necessitates the identification of new additional risk factors to predict and assess the risk of acute coronary syndromes[89, 90].

Studies have shown that there is an association between inflammation and cardiovascular disease [83, 91, 92, 93]. Inflammation plays a central role from initiation to development of clinical complication in the pathogenesis of atherosclerosis from formation of fatty streak to plaque rupture[94]. Studies showed that individuals with myocardial infarction showed a elevated serum CRP levels and the levels tend to remain elevated in complicated cases[95]. The baseline CRP levels predicted the future risk for the development of myocardial infarction or stroke in apparently healthy men[96]. Results from the women's health study showed that eventhough other markers of inflammation like serum amyloid A (SAA), interleukin-6 (IL-6) and soluble intracellular adhesion molecule were also measured, serum CRP level has been proved to be a strong independent predictor of risk of cardiovascular events[97].

High sensitivity CRP (Hs-CRP)

Individuals with increased CRP levels are at 2 to 4 times higher risk of developing cardiovascular events. To predict risk of cardiovascular events among apparently healthy individuals high sensitivity assays are developed to detect CRP levels less than 10mg/l, the CRP levels detected by these "high-sensitivity" assays are called hs-CRP[80].

Hs-CRP assays have been developed with acceptable precisions less than 0.3 mg/L, these previous normal ranges for CRP , predicted by hs-CRP assays seem to have the ability to identify CVD events[93].

Large population based studies like the MONICA study[98] , the Women's health study[99] showed that there exists a dose-response relationship between the levels of hs-

CRP and risk of incidence of coronary events. Hs-CRP evaluation added to other established risk factors increased the predictive capacity of coronary events. Studies showed that through stratification, hs-CRP retains an independent association with incident coronary events after adjusting for age, total cholesterol, HDL cholesterol, smoking, body mass index, diabetes, history of hypertension, exercise level, and family history of coronary disease[88, 96]. Hs-CRP consistently predicts new coronary events in patients with unstable angina and acute myocardial infarction[93].

Prediction levels / Interpretation of hs-CRP assays

Hs-CRP levels can range between 0.3 to 8.6 mg/L for healthy men and 0.2 to 9.1 mg/L for healthy women who are not in hormone replacement therapy and at these low levels hs-CRP is able predict future coronary events in apparently healthy people[100]. The centre for disease control (CDC) and American heart association (AHA) recommended the following interpretation of hs-CRP results:

- < 1 mg/L : Low risk
- 1to 3 mg/L: Average risk
- > 3 mg/L: High risk.

An hs-CRP level of >10 mg/L, for example, should be repeated in 2 weeks to allow acute inflammations to subside before retesting, two separate measurements of hs-CRP are adequate to classify a person's risk level and to account for the increased within-individual variablility[101] .There is absence of diurnal variation in CRP levels[102]

Other sources for variation of CRP levels, include obesity and metabolic syndrome increase hs-CRP levels and loss of weight seem to coincide with reduction in weight[93].

Inflammatory markers may have greater potential as a means to augment risk assessment in the identification of persons who should be considered lipid-lowering, antiplatelet, or other cardioprotective drug therapies, as well as for increased emphasis on therapeutic lifestyle changes.The potential utility of hs-CRP is thus to target patients for primary intervention[93].

CRP in saliva

In a study[103] (n= 55) to detect Homocysteine and CRP levels in saliva of healthy adults and comparison with blood levels showed that when CRP concentrations were determined using ELISA. CRP was measurable in all saliva samples (range: 0.05 to 64.3 µg/L; median = 1.2 µg/L) and plasma samples (range: 0.14 to 31.1 mg/L; median = 2.0 mg/L). Regression analysis demonstrated no relationship between CRP concentration in saliva and plasma ($R^2 = 0.001$).

In a study by Niveditha. L. Rao *et al*[67] to estimate and compare salivary CRP levels between patients with Hashimato's thyroiditis (HT, n=30) and subacute thyroiditis (SAT, n=15) with 20 healthy gender and age matched controls, the salivary CRP levels are evaluated using ELISA with enhanced sensitivity (salimetrics). The results showed that the salivary CRP levels are not significantly different from controls in HT group. SAT patients had significantly increased salivary CRP levels when compared to both HT and control group and this increase reflects the presence of inflammatory process in SAT and salivary CRP may aid in diagnosis and clinical evaluation of SAT.

In a Finland study[59] done utilizing cross-sectional data from 250 coronary artery disease (CAD) and 250 non- CAD patients, to determine the association of salivary lysozyme (SLZ) and CRP with metabolic syndrome (metS) which is a pro

inflammatory state, salivary CRP was determined by high-sensitivity immunoturbidometry assay. Salivary CRP levels were distinctly higher in the CAD group than in the non-CAD group regardless of metS status and CRP was not significantly associated with metS independent of CRP.

In a paper published by Christadoudiles N *et al*[77]. a novel method was used to detect the CRP in human saliva this newer lab-on-chip technique based on electronic taste chip (ETC) approach showed higher sensitivity than the traditional hs-CRP ELISA, because of the increased surface area of the micro beads for more antigen/antibody interaction, and was able to detect CRP levels as low as 10 picograms/ml even with 1000 fold of dilution of saliva. This study showed that higher values of CRP were seen in subjects with chronic periodontal disease.

A feasibility study by Floriano PN *et al*[7]. in the use of saliva based nano-biochip for acute myocardial infarction. A Luminex and lab-on-a-chip methods was used to assay 21 proteins in serum and unstimulated whole saliva procured from 41 AMI patients within 48 h of chest pain onset and from 43 apparently healthy controls.. The results showed significant difference in concentrations of cardiac biomarkers both traditional (Creatinine kinase, myoglobin, cardiac troponin I) and novel (CRP, Myeloperoxidase) in subjects with AMI and healthy controls.

Foley JD *et al*[104] conducted a study to determine the ability of the salivary biomarkers to identify different aspects of myocardial necrosis. In this study serum and UWS was procured from patients undergoing alcohol septal ablation (ASA) for treatment of hypertrophic cardiomyopathy at baseline and incremental time points post-ASA. Seven biomarkers related to myocardial damage, inflammation, and tissues remodelling using immunosorbent assays were analyzed. Levels were compared with

baseline and levels observed in 97 healthy controls. The results showed biomarkers of myocardial damage and inflammation (ie, troponin I, creatine kinase-MB, myoglobin, C-reactive protein) rose in serum upto 2- to 812-fold after ASA .Significant elevations of 2.0- to 3.5-fold were observed with CRP and troponin I in saliva. Significant correlations between levels in serum and saliva were observed for C-reactive protein, matrix metalloproteinase-9, and myeloperoxidase. These data suggest that select salivary biomarkers can reflect the changes in myocardial necrosis that is caused by ASA which occurs during and after the treatment.

A study by Punyadeera C *et al*[78] using a homogeneous bead based assay to detect CRP levels in human saliva was done to establish a saliva CRP reference range and to demonstrate the clinical utility of salivary CRP levels in assessing the coronary events in a primary health care setting. Saliva from cardiac patients and healthy controls were assessed by means of a one step optimized assay, and the results showed a positive correlation between serum and saliva samples and the salivary CRP levels from healthy volunteers and cardiac patients were distinguishable.

Immunoturbidimetric assay for quantitative detection of Hs-CRP

Different methods for quantifying CRP in serum have been used. Immunoturbidimetric (IT) assay is used to detect CRP through high sensitive assay. This technique measures increased turbidity resulting from antigen-antibody complexes. The technique uses ready to use liquid reagents and a chemical analyser which can measure the absorbance values and results are obtained within 20 minutes.

Robert *et al*[105] evaluated nine hs-CRP assays (7- IT; 1- Immunonephelometric (IN); and 1- Immunoluminometric (IL)) from nine different manufacturers for limit of detection, linearity, precision, prozone effect, and comparability with samples from

388 apparently healthy individuals. Of the nine methods evaluated, seven had limits of detection < 0.2 mg/L ,therefore the paper concluded that hs-CRP assays has to be standardized for concentrations of 0.2–10 mg/L so that results obtained in large population studies can be applied to individual patients cut-off-points and with establishment of standardization, hs-CRP assays can provide useful data for coronary risk stratification in apparently healthy individuals.

In a study by Kusnierz-Cabala B *et al*[106], which compared hs-CRP assays using nephelometry and immunoturbidimetric techniques to evaluate the serum CRP concentrations to assess the cardiovascular risk in chronic metabolic diseases. The results were similar with techniques and it was concluded that turbidimetric high sensitive CRP assays can properly classify CRP-related prediction of chronic metabolic diseases with special consideration on cardiovascular risk.

MYOGLOBIN

Functions and role in clinical diagnosis

Myoglobin is a heme protein (Molecular weight = 17.8Kda) and helps in transport of oxygen within muscle cells.Myoglobin constitutes about 2 % of muscle protein in both cardiac and skeletal muscle. Because of its low molecular weight, myoglobin is rapidly released into the circulation and is the early marker to rise after an AMI and it was the first serum cardiac marker that was not an enzyme or an isoenzyme.

Serum myoglobin levels are elevated in conditions unrelated to AMI, such as skeletal muscle, neuromuscular disorders, renal failure, intramuscular injection, strenuous exercise and in presence of various toxins and drugs Very little free myoglobin (12-100ng/ml) circulates as result of natural protein turnover.

During the course of a myocardial infarction, myoglobin escapes from the ischaemic cardiac muscle, and the myoglobin levels increase measurably above baseline within 2-4 hours post-infarct, peaking at 9-12 hours, and returning to baseline within 24-36 hours It is rapidly removed from circulation, filtered through the glomerular membrane of the kidney, and excreted in the urine. Rapid kinetic of myoglobin serum level is important for its utility as marker for reperfusion and re-infarction diagnosis[12,107].

In a study by McComb JM *et al* 1984[108], to compare the sensitivity and specificity of the serum levels of creatinine kinase (CK) and myoglobin to diagnose AMI, it was found that the serum myoglobin concentration was raised (> 85g/l) in 25% of patients at one hour of onset of symptoms and in 89% of patients at four hours, compared to 25% and 56% of serum CK levels respectively, within 12 hours the myoglobin values peaked in 83% of the patients compared to only 14% of CK and the myoglobin values fell to normal within 36 hours in 36 % while the CK values came back to normal in only 4 % of patients at 36 hours. This study showed that a single measurement of serum myoglobin concentration at 6 hours from onset of symptoms predicts diagnosis of suspected myocardial infarction with a sensitivity of 93% and specificity of 89% .

In a study[109] to evaluate the value of myoglobin, CK-MB $_{mass}$ and TroponinT to rule out AMI in the emergency department, it was found that myoglobin is the earliest marker and has got 89% negative predictive value (NPV) after 4 hours of onset of symptoms and this is significantly higher than the NPVs of CK-MB $_{mass}$ and TroponinT. Therefore myoglobin is a better marker to rule out AMI in the emergency room from 3 to 6 hours of onset of symptoms.

A study by McCord J *et al*[110] evaluated a serum multimarker stratergy including Creatinie kinase –MB (CK-MB), Myoglobin and cardiac troponin I using a point-of-care quantitative detection to rule out AMI in ≤ 3 hours. In patients admitted to the emergency department with signs and symptoms of AMI, serum was collected from patients with non diagnostic ECGs, and the CK-MB, troponin I, and myoglobin levels were measured with a point-of-care device at presentation and at 90 minutes, 3 hours, and 9 hours. The results of this study concluded that AMI can be "ruled out" or "ruled in" more rapidly by use of a combination of normal cTnI and myoglobin values done at 0 and 90 minutes after admission in the emergency department.

Myoglobin in saliva

In a study[51] to evaluate the elevated levels of salivary proteins like immunoglobulin E, nerve growth factor and myoglobulin by means of ELISA from unstimulated whole saliva collected from patients with type 2 diabetes mellitus and autoimmune diseases and normal controls, it is found that the salivary levels of the above mentioned protein markers were raised in type 2 diabetes and other diseased patients than in normal controls.

In a poster presented by Yashari J *et* al[111] at the AADR annual meet 2010, the relationship between salivary and serum biomarkers in AMI-patients and non-AMI controls to determine the ability of the salivary and serum biomarkers to discriminate between different types (STEMI and NSTEMI) of AMI was studied. In this study (UWS) whole saliva and serum were obtained from controls and AMI patients within 72 hours of chest pain onset. Levels of brain-natriuretic peptide (BNP), troponin I (TnI), creatine kinase-MB (CK-MB), myoglobin (MYO) and 13 Luminex-analyzed biomarkers relevant to cardiovascular disease were determined in the serum and saliva.

The results showed that MYO levels in UWS were significantly different within 48 hours of chest pain onset in AMI patients compared with non-AMI patients, and UWS MYO levels correlated positively ($r=0.55$) with serum concentrations ($p<0.0001$). Significant differences in biomarker profiles between ST elevated MI and non-ST elevated MI were observed in serum, but not UWS. From these findings it can be suggested that that select UWS biomarkers may be useful for aiding in the identification of AMI.

C-reactive protein and Myoglobin in diagnosis of AMI:

A study[112] was done to investigate the sensitivity and specificity of myoglobin to diagnose MI in emergency room and also to investigate the relationship of serum CRP and MYO together in early diagnosis of MI. Samples were collected on admission and after 6 hours. The results showed that the serum concentration of MYO was higher in all MI patients with 100% sensitivity but it was also higher in 20% of normal controls making the specificity 78%, the serum CRP levels were also higher in MI patients than normal controls. The results of the study showed that the MYO levels should assessed atleast twice once on admission and one after 6 hours and the CRP levels when assessed on the first day of admission render a faster and better diagnoses.

CRP and MYO in saliva

Floriano PN *et al*[7] observed that the saliva-based biomarker panel of C-reactive protein, myoglobin, and myeloperoxidase exhibited significant diagnostic capability and in conjunction with ECG yielded strong screening capacity for AMI, so the saliva based nano- bio chip can be used as an adjunct in diagnosis of AMI along with ECG in prehospital stages.

Yashari J *et al*[111] in their study using UWS for evaluation of salivary biomarkers in AMI found that, UWS levels of MYO and CRP were raised in AMI patients and showed positive correlation with serum concentrations (r=0.55 and 0.37 respectively).

Results

Out of the 30 samples collected from Group I subjects, only 28 samples were used. The 2 samples were excluded because the 24-48hours and 48-72hours sample could not be collected as the patients developed pneumonia. Among the 28 samples for 4 samples the 48-72 hours sample could not be collected because the patient was shifted to a different medical facility. Only 24 subjects had all three saliva samples.

Out of the 15 samples collected from Group II A subjects, only 14 samples were used, one sample was excluded because of the viscosity detected at the time of analysis. Out of the 14 samples used in Group II A 2 samples were not included in the results as they showed abnormal (outliers) values

Out of the 15 samples collected from Group II B subjects, only 13 samples were used, 2 samples were excluded because of sample contamination detected at the time of analysis.

The study population consists of 3 groups, Group I – Acute myocardial infarct patients (n=28) Group II A- controls with periodontitis (n=12) and Group II B- apparently healthy controls without periodontitis (n=13). In group I 60.7% (n=17, males=17, females=0) had ST segment elevated myocardial infarction (STEMI) and 30.9% (n=11, males=6, females=5) had Non-ST segment elevated myocardial infarction (NSTEMI).

Gender distribution among study groups (Table 1; Graph 1)

The distribution of gender among the study population was 82.1% of males (n=23) and 17.9% of females (n=5) in Group I, 50% males (n=6) & 50% females (n=6) in Group II A and 30.8% males (n=4) and 69.2% of females (n=9) in Group II B.

Age distribution among study groups (Table 2; Graph 2)

The mean age in Group I was 54.8 ± 9.6 (males=56.1 ± 9, females=48.8 ± 11.1) with an age range of 35-71 years. The mean age in Group II A was 47.3 ± 9.9 (males=50 ± 11.4, females= 44.6 ± 9.8) with an age range of 35 - 63 years. The mean age in Group II B was 44.6 ± 9.6 (males=50.7 ± 11.5, females=41.8 ± 7.9) with an age range of 34 - 65 years.

Prevalence of habits among the study groups (Table 3; Graph 3)

In group I 82.1 % (n=23) of individuals had no habit of tobacco smoking or drinking alcohol, 14.3 % (n=4) had the habit of cigarette smoking and 3.6 % (n=1) had the habit of drinking alcohol. In group II A 75% of individuals had no habit of tobacco smoking or drinking alcohol and 25% had the habit of smoking cigarettes. In group II B none had the habit of tobacco smoking or drinking alcohol. The prevalence of habits are not statistically significant among the study groups (P=0.533)

Expression of salivary Hs-CRP levels (mg/L) between study groups (Tables 4 to 8; Graphs 4 to 6)

The salivary hs-CRP levels are higher in Group I when compared to Group II A and Group II B in samples collected at 6-12 hours (P=0.255), 24- 48 hours (P=0.677) and 48- 72 hours (P=0.266). Multiple comparisons between groups I, II A, II B with ANOVA followed by Tukey HSD didn’t show any statistical significant difference between groups in salivary hs-CRP expression. Comparison between Group I and Group II (Gp II A+ II B) by t-Test didn’t show any statistically significant difference in salivary expression level of hs-CRP in samples collected at 6-12hours

(P=0.157), 24-48hours (P=0.527) and 48-72hours (P=0.177). There is no statistically significant difference among the salivary hs-CRP levels collected at 6-12hours, 24-48hours and 48-72hours from patients in Group I (P=0.113). Salivary hs-CRP levels between STEMI and NSTEMI patients in Group I at samples collected at 6-12hours (P=0.132), 24- 48 hours(P=0.241) and 48-72 hours(P=0.379) are not statistically significant.

In group I out of the 28 subjects from whom the samples were collected the 48 -72 hours sample was not collected from subjects 11, 15, 16 and 28 and for subject 14 the 6 -12 hours sample was not sufficient. (Graph 4). All the saliva samples showed values of hs-CRP in the range of 0.2 mg/L to 1.47mg/L. Among the 28 samples, sample 4 and 21 showed increased expression (1.47mg/L and 1.45mg/L respectively) in the samples collected at 24- 48 hours compared to samples collected at 6-12hours (0.76mg/L and 0.65mg/L) and samples collected at 48 -72 hours (0.59mg/L and 0.6mg/L) samples 9 and 27 showed increased expression (1.28mg/L) in samples collected at 48 – 72 hours compared to samples collected at 6-12hours (0.44mg/L and 0.15mg/L respectively) and samples collected at 24-48 hours (0.46mg/L and 0.79mg/L).

In group II A all the 12 samples showed salivary hs-CRP expression the values ranged from 0.24 mg/L to 0.69 mg/L (graph 5). In group II B all the 13 samples showed salivary hs-CRP expression the values ranged from 0.35mg/L to 0.87mg/L. (Graph 6)

Expression of salivary Myoglobin (ng/ml) between groups (Tables 9-13; Graphs 7-9)

Salivary Myoglobin levels are higher in Group II B than Group II A, which was in turn higher than the Group I in samples collected at 6 -12 hours (P=0.126) and this difference was statistically significant in samples collected at 24- 48 hours (P=0.013**)** and 48-72 hours (P=0.022**)**. Multiple comparisons between Group I, II A and II B with Tukey HSD showed no statistically significant difference in salivary myoglobin expression in samples collected at 6-12 hours.

On comparison between samples collected at 24-48hours in Group I subjects and Group II A and II B, there is no difference in the salivary myoglobin expression between Group I and II A. The salivary myoglobin expression showed a significant increase in Group II B than in Group I (P=0.010).

On comparison between samples collected at 48-72hours in Group I subjects and Group II A and II B, there is no difference in the salivary myoglobin expression between Group I and II A. The salivary myoglobin expression showed a significant increase in Group II B than in Group I (P=0.017).

t-test was done to compare the salivary myoglobin expression between Group I and Group II (Group II A + II B), there is no difference in samples collected at 6-12hours. The expression levels showed significant increase in samples collected at 24-48hours (P=0.009) and 48-72hours (P=0.015), the expression higher in Group II than in Group I.

Expression of salivary myoglobin level within Group I showed a significant increase in the samples collected at 6 - 12 hours (P=0.018) than samples collected at 24 - 48 hours and 48 - 72hours.

Expression of salivary myoglobin levels between STEMI and NSTEMI patients in Group I in samples collected at 6 -12 hours was significantly increased (P=0.043), the expression being higher in NSTEMI patients and not significant in samples collected at 24 - 48hours (P=0.553) and 48 - 72hours sample (P=0.545).

In group I out of the 28 subjects from whom the samples were collected, the 48 -72 hours sample was not collected from subjects 11, 15, 16 and 28. Out of the 28 samples, samples 4, 6, 7, 8,9, 11, 13, 14, 15, 17, 18, 20, 21, 23, 24, 26 & 28 expressed negative (below the absorbance levels) values , in subject 6 and 17 all the 3 samples collected at 6 -12 hours, 24- 48 hours and 48 – 72 hours expressed negative values and in subject 11, 15 and 28 both the samples collected at 6 -12 hours and 24-48 hours showed negative values (Graph 7). Out of the 28 samples 14 expressed increased salivary myoglobin values in the samples collected at 6 – 12 hours compared to samples collected at 24 – 48 hours and 48 – 72 hours, 2 samples showed a mild increase in values in the sample collected at 24- 48 hours compared to 6-12hours and 48- 72 hours sample and 2 samples showed increase in myoglobin expression in sample collected at 48 – 72 hours. The salivary myoglobin values ranged from 0.154 ng/ml to 10.23 ng/ml.

In group II A out of the 12 samples, samples 5 and 10 showed negative values for salivary myoglobin expression. The values ranged from 0.208 ng/ml to 10.23 ng/ml (Graph 8). In group II B out of the 13 samples, samples 3 and 12 showed negative values salivary myoglobin expression. The values ranged from 0.917ng/ml to 11.63ng/ml. (Graph 9).

Tables and Graphs

Table 1
Gender distribution among study groups

Study Groups	Male	Female
Group I (n = 28)	n = 23 82.1%	n = 5 17.9%
Group II A (n = 12)	n = 6 50 %	n = 6 50 %
Group II B (n = 13)	n = 4 30.8%	n = 9 69.2%

Graph 1
Gender distribution among study groups

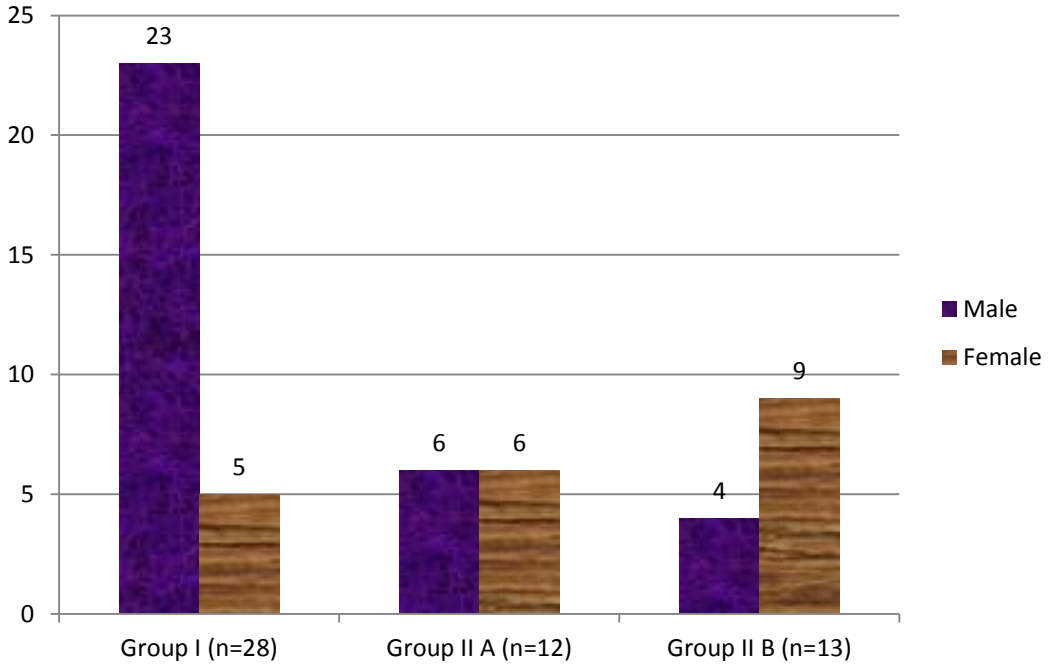


Table 2
Age distribution (in years) among study groups

Study Groups	Overall Mean Age	Mean Age of Males	Mean Age of Females
Group I (n = 28, M = 23, F=5)	54.8 ± 9.6	56.1 ± 9	48.8 ± 11.1
Group II A (n = 12, M = 6, F=6)	47.3 ± 9.9	50 ± 11.4	44.6 ± 9.8
Group II B (n = 13, M = 4, F=9)	44.6 ± 9.6	50.7 ± 11.5	41.8 ± 7.9

Graph 2
Distribution of Age (in years) among study groups

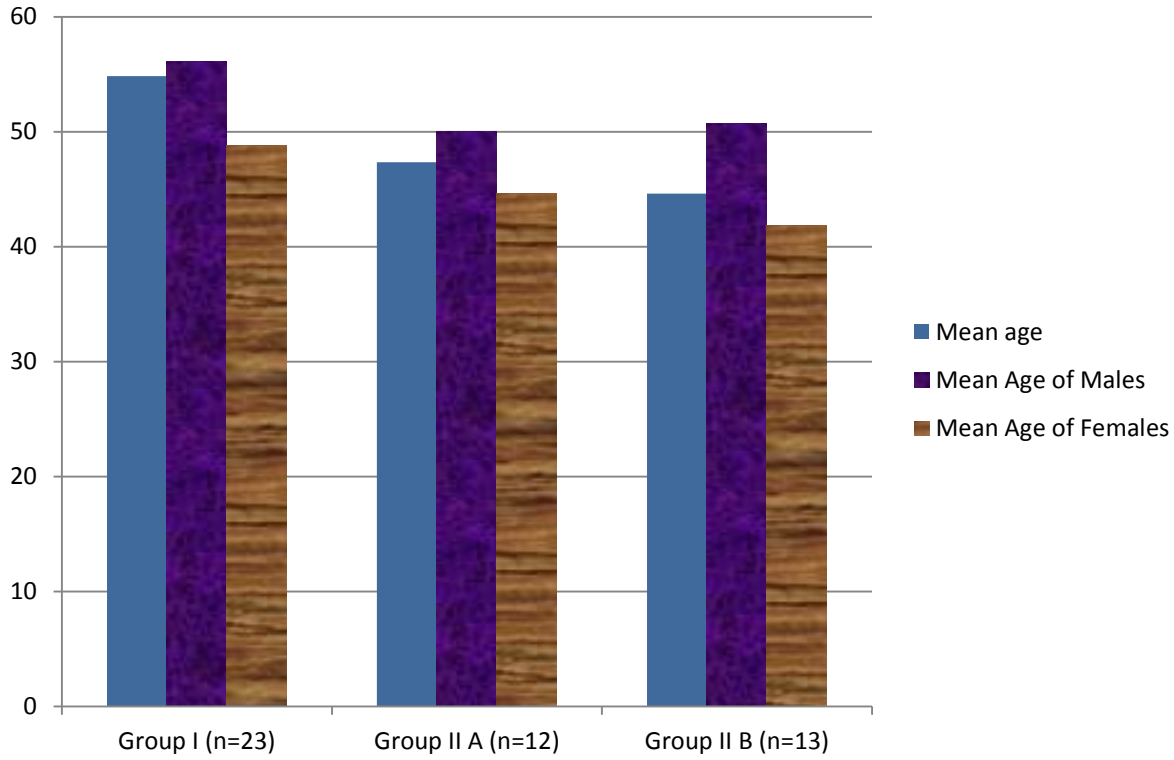


Table 3
Prevalence of habits among study groups

Study Groups	No Habit	Smoking	Drinking
Group I (n = 28)	n = 23 82.1%	n = 4 14.3%	n = 1 3.6%
Group II A (n = 12)	n = 9 75%	n = 3 25%	n=0
Group II B (n = 13)	n = 13 100%	n = 0	n = 0

Graph 3
Prevalence of habits among study groups

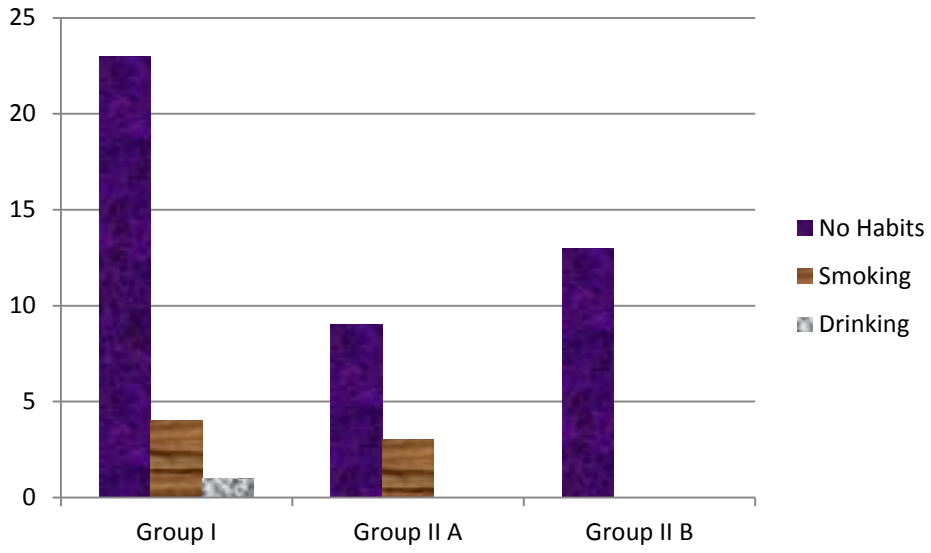


Table 4
Expression of salivary hs-CRP (mg/L) between groups

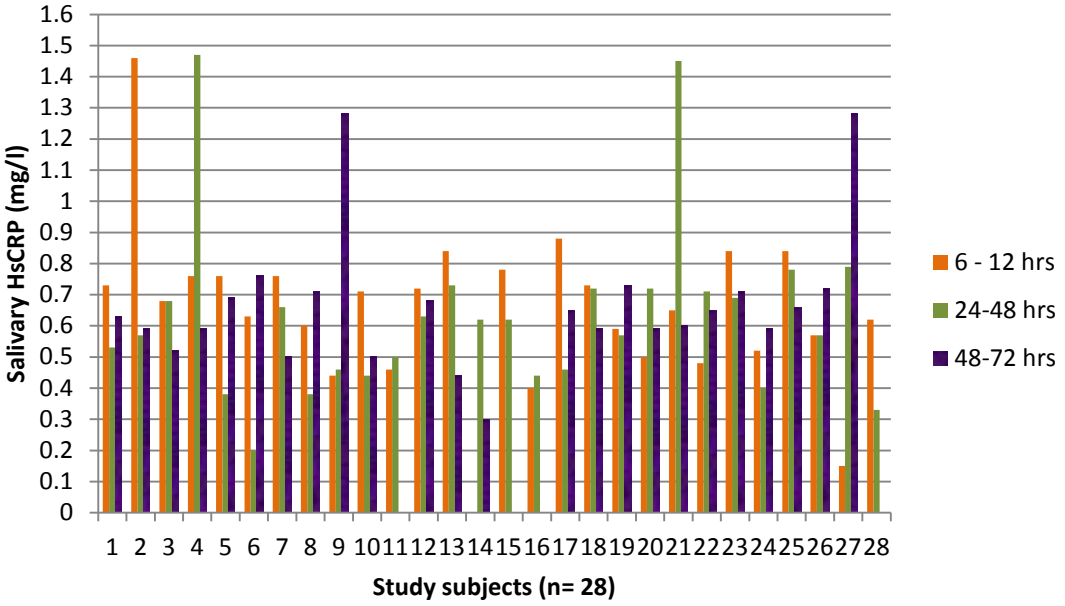
		n	Mean	Std. Deviation	P value
Saliva-HS-CRP(mg/L) 6-12hrs	Group I	27*	.67037	.228397	0.255
	Group II A	12	.56083	.130346	
	Group II B	13	.62692	.132249	
Saliva-HS-CRP(mg/L) 24-48hrs	Group I	28	.62500	.277936	0.677
	Group II A	12	.56083	.130346	
	Group II B	13	.62692	.132249	
Saliva-HS-CRP(mg/L) 48- 72hrs	Group I	24**	.66500	.215790	0.266
	Group II A	12	.56083	.130346	
	Group II B	13	.62692	.132249	

***-** Sample collected at 6-12 hours was not used as it was not sufficient.

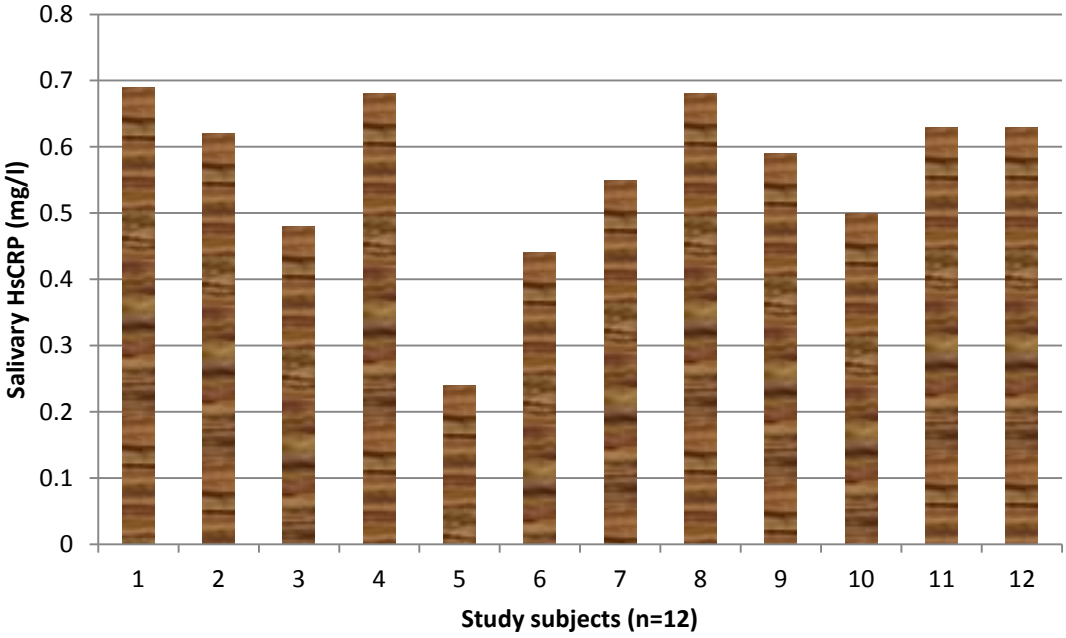
**- 3rd sample at 48-72 hours could not be collected

Graph 4

Salivary hs-CRP expression (mg/L) in Group I



Graph 5
Salivary hs-CRP expression (mg/L) in Group II A



Graph 6
Salivary hs-CRP expression (mg/L) in Group II B

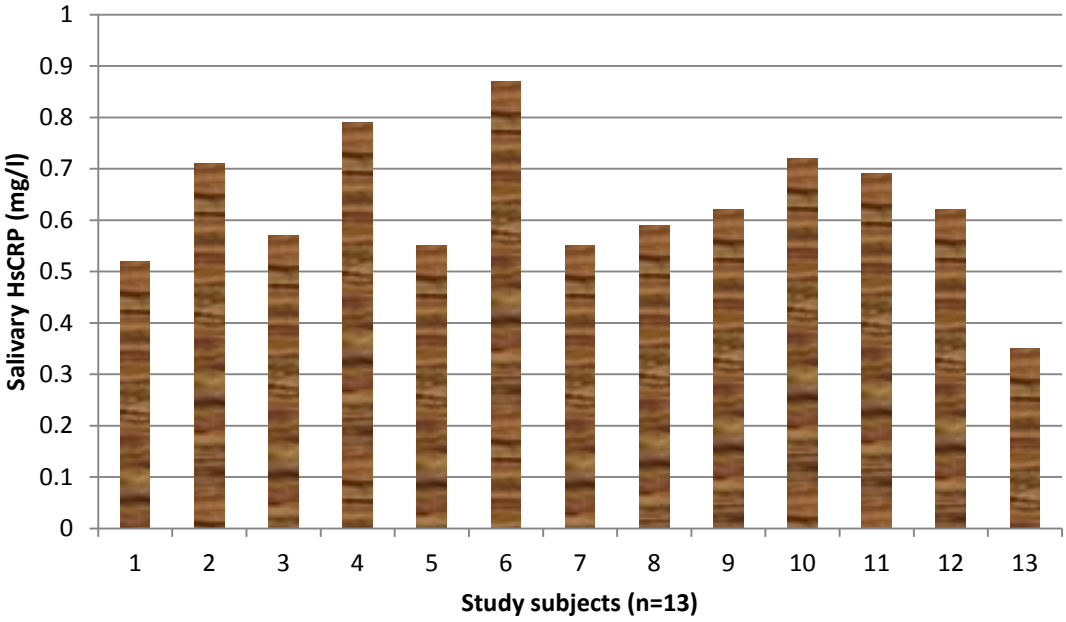


Table 5

Multiple comparison of salivary hs-CRP expression between Group I, II A and II B

Dependent Variable	(I) Group	(J) Group	Mean Difference (I-J)	P value
Saliva-hs-CRP(mg/L) 6 -12hrs	Group I	Group II B	.04345	0.776
		Group II A	.10954	0.227
	Group II B	Group I	-.04345	0.776
		Group II A	.06609	0.660
	Group II A	Group I	-.10954	0.227
		Group II B	-.06609	0.660
Saliva-hs-CRP(mg/L) 24 – 48hrs	Group I	Group II B	-.00192	1.000
		Group II A	.06417	0.683
	Group II B	Group I	.00192	1.000
		Group II A	.06609	0.740
	Group II A	Group I	-.06417	0.683
		Group II B	-.06609	0.740
Saliva-hs-CRP(mg/L) 48- 72hrs	Group I	Group II B	.03808	0.810
		Group II A	.10417	0.236
	Group II B	Group I	-.03808	0.810
		Group II A	.06609	0.628
	Group II A	Group I	-.10417	0.236

Table 6
Expression of salivary hs-CRP between Group I and Group II (II A + II B)

	Group	n	Mean	Std. Deviation	P value
Saliva-hs-CRP(mg/L)6 - 12hrs	Group I	27*	.67037	.228397	0.157
	Group II	25	.59520	.132920	
Saliva-hs-CRP(mg/L)24 – 48hrs	Group I	28	.62500	.277936	0.627
	Group II	25	.59520	.132920	
Saliva-hs-CRP(mg/L)48 – 72hrs	Group I	24**	.66500	.215790	0.177
	Group II	25	.59520	.132920	

***-** Sample collected at 6-12 hours was not used as it was not sufficient.

**- 3rd sample at 48-72 hours could not be collected

Table 7
Expression of salivary hs-CRP level within Group I

	n*	Mean	Std. Deviation	P value
Saliva-hs-CRP(mg/L) 6-12hrs	23	.68870	.235165	
Saliva-hs-CRP(mg/L) 24-48hrs	23	.65174	.296367	0.113
Saliva-hs-CRP(mg/L) 48-72hrs	23	.68087	.205822	

***-** Only cases with all three values were included.

Table 8
Comparison of hs-CRP expression in saliva between
STEMI and NSTEMI patients in Group I

	ECG	n	Mean	Std.deviation	P value
Saliva-hs-CRP(mg/L) 6 -12hrs	STEMI	16*	.72563	.240582	0.132
	N STEMI	11	.59000	.191885	
Saliva-hs-CRP(mg/L) 24 – 48hrs	STEMI	17	.57471	.266649	0.241
	N STEMI	11	.70273	.289589	
Saliva-hs-CRP(mg/L) 48 – 72hrs	STEMI	14**	.63143	.222982	0.379
	N STEMI	10**	.71200	.207300	

STEMI: ST elevated myocardial infarction, NSTEMI: Non-ST elevated myocardial infarction

***-** Sample collected at 6-12 hours was not used as it was not sufficient.

**- The 3rd sample at 48-72 hours could not be collected

Table 9

Expression of salivary Myoglobin (ng/ml) between groups

		n	Mean	Std.deviation	P value
Saliva-MYO(ng/ml) 6 -12hrs	Group I	28	1.43589	3.615417	0.126
	Group II A	12	1.67508	3.718136	
	Group II B	13	4.40246	6.134160	
Saliva-MYO(ng/ml) 24- 48hrs	Group I	28	-.60568#	4.713742	0.013*
	Group II A	12	1.67508	3.718136	
	Group II B	13	4.40246	6.134160	
Saliva-MYO(ng/ml) 48- 72hrs	Group I	24**	-.64842#	5.117443	0.022*
	Group II A	12	1.67508	3.718136	
	Group II B	13	4.40246	6.134160	

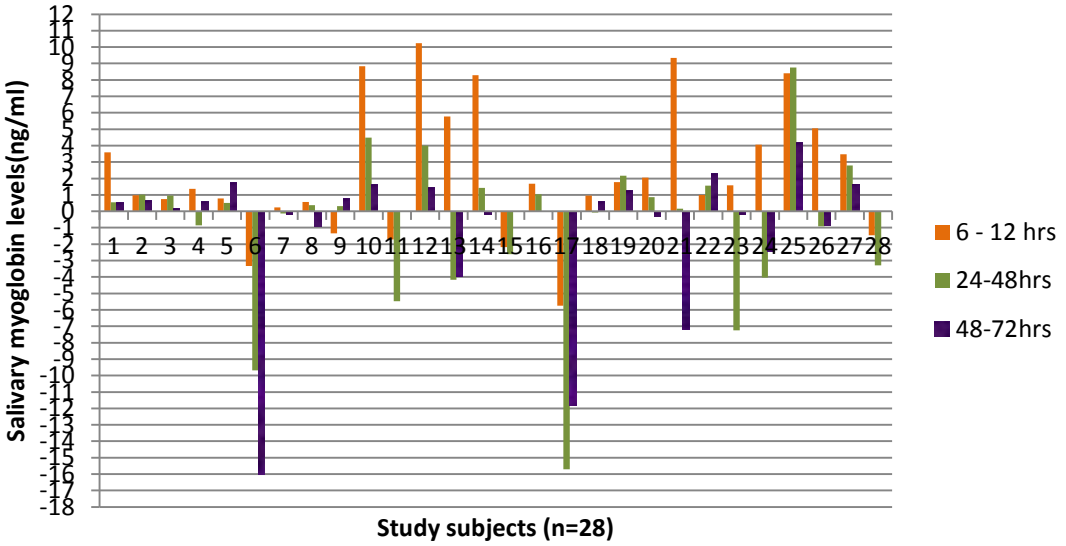
* P value is significant at the level of 5%, P<0.05

The mean values are negative as 60% of the samples showed negative (below the absorbance level) values.

**- 3rd sample at 48-72 hours could not be collected

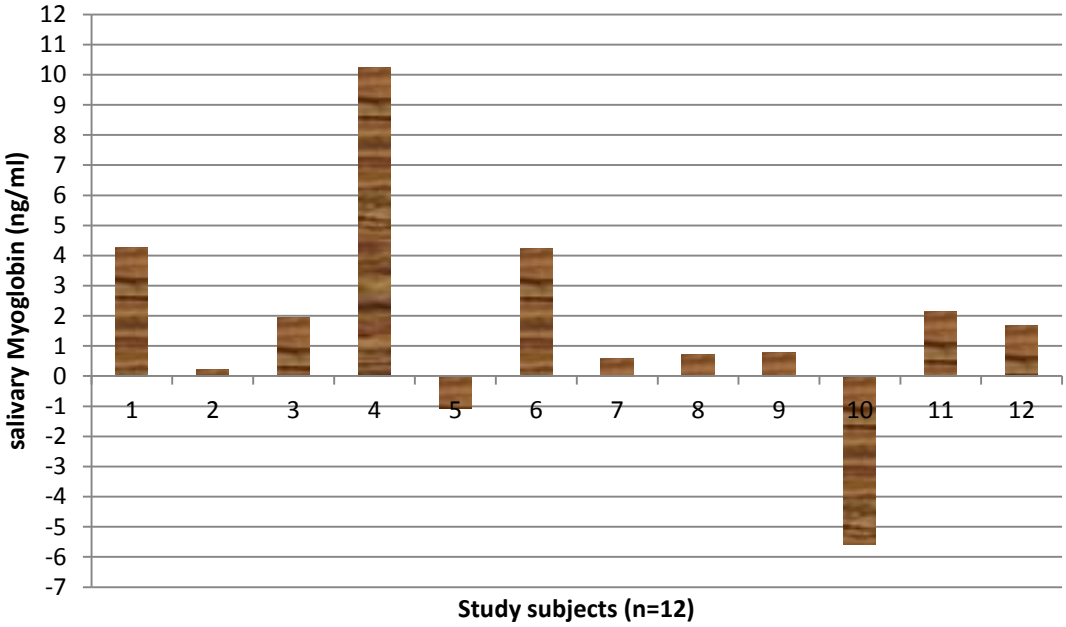
Graph 7

Salivary myoglobin expression (ng/ml) in Group I



Graph 8

Salivary myoglobin expression (ng/ml) in Group II A



Graph 9

Salivary myoglobin expression (ng/ml) in Group II B

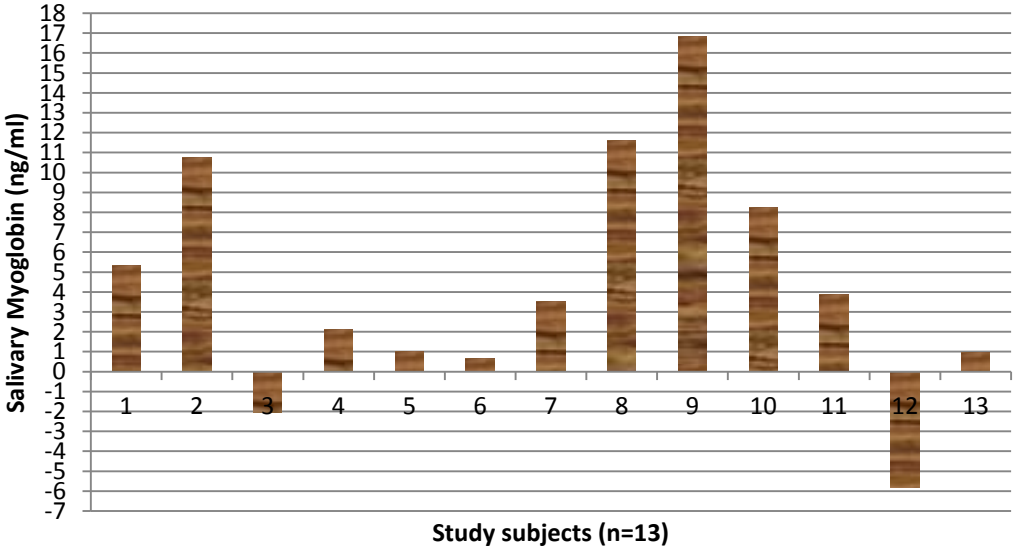


Table 10

Multiple comparison of salivary Myoglobin levels among groups

Dependent Variable	(I) Group	(J) Group	Mean Difference (I-J)	P value
Saliva-MYO(ng/ml) 6-12hrs	Group I	Group II B	-2.96657	0.118
		Group II A	-.23919	0.986
	Group II B	Group I	2.96657	0.118
		Group II A	2.72738	0.273
	Group II A	Group I	.23919	0.986
		Group II B	-2.72738	0.273
Saliva-MYO(ng/ml) 24- 48hrs	Group I	Group II B	-5.00814	0.010*
		Group II A	-2.28076	0.376
	Group II B	Group I	5.00814	0.010
		Group II A	2.72738	0.354
	Group II A	Group I	2.28076	0.376
		Group II B	-2.72738	0.354
Saliva-MYO(ng/ml) 48- 72hrs	Group I	Group II B	-5.05088	0.017*
		Group II A	-2.32350	0.412
	Group II B	Group I	5.05088	0.017
		Group II A	2.72738	0.386
	Group II A	Group I	2.32350	0.412
		Group II B	-2.72738	0.386

* P value is significant at the level of 5%, P<0.05

Table 11

Expression of salivary Myoglobin levels between Group I and Group II

(II A+ II B)

	Group	n	Mean	Std. Deviation	P value
Saliva-MYO(ng/ml) – 6-12hrs	Group I	28	1.43589	3.615417	0.180
	Group II	25	3.09332	5.204252	
Saliva-MYO(ng/ml) – 24-48hrs	Group I	28	-.60568	4.713742	0.009*
	Group II	25	3.09332	5.204252	
Saliva-MYO(ng/ml) – 48-72hrs	Group I	24**	-.64842	5.117443	0.015*
	Group II	25	3.09332	5.204252	

**- 3rd sample at 48-72 hours could not be collected

Table 12

Expression of salivary Myoglobin level within Group I

	n**	Mean	Std. Deviation	Minimum	Maximum	
Saliva-MYO(ng/ml) 6-12hrs	24	1.82746	3.717651	-5.740	9.346	P=0.018*
Saliva-MYO(ng/ml) 24-48hrs	24	-.27575	4.933014	-15.700	8.755	
Saliva-MYO(ng/ml) 48-72hrs	24	-.64842	5.117443	-16.000	10.230	

* P value is significant at the level of 5%, P<0.05

**- Only cases with all three sample values were included.

Table 13

Comparison of Myoglobin expression in saliva between STEMI and NSTEMI patients in Group I

	ECG	n	Mean	Std. Deviation	P value
Saliva-MYO(ng/ml) – 6-12hrs	STEMI	17#	.33794	3.401416	0.043*
	N STEMI	11	3.13273	3.400649	
Saliva-MYO(ng/ml) – 24-48hrs	STEMI	17	-1.04171	5.117058	0.553
	N STEMI	11	.06818	4.156289	
Saliva-MYO(ng/ml) – 48-72hrs	STEMI	14**	-1.19843	6.224650	0.545
	N STEMI	10**	.12160	3.135162	

* P value is significant at the level of 5%, P<0.05

#- Sample collected at 6-12 hours was not used as it was not sufficient.

**- 3rd sample at 48-72 hours could not be collected

Figures

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Fig 1. CS 400 Auto analyser – Hs-CRP IT assay

Fig 2. Micro-centrifuge tube (1.5ml)

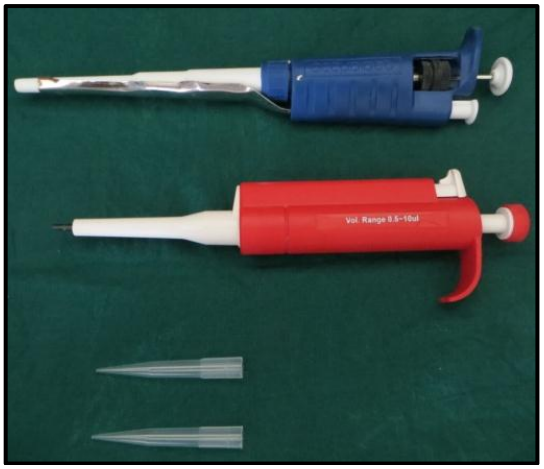

Fig 3. Micropipettes and pipette tips

Fig 4. Myoglobin ELISA reagents

Fig 6. ELISA-Auto washer

Fig 5. ELISA- microtitre well reader

Discussion

This study was done to evaluate saliva as a diagnostic tool in early diagnosis of acute myocardial infarction (AMI) by quantitatively detecting the levels of C-reactive protein by means of a high-sensitivity immunoturbidimetric assay and Myoglobin by ELISA and to compare it with non-AMI controls.

Human saliva contains proteins that are present in serum and its collection is non-invasive which makes it an ideal tool for large patient screening and diagnosis[1,78].

Myocardial infarction is a major cause of death and disability worldwide in both gender in the adult age group of 15-59 years[69, 8,73]. Cross-sectional studies in India have shown a rising trend in Coronary artery disease (CAD) in the past 30 years and a higher prevalence is seen in South India[72, 73].

The prevention and treatment of CAD requires a highly responsive healthcare system, which includes use of novel and newer biomarkers and techniques which can aid in screening, early diagnosis and predict future cardiovascular events in apparently healthy people.

CRP is an acute phase reactant which is released in circulation during the inflammatory and infectious conditions. CRP levels when assessed by high-sensitive assays helped in detection of future risk of cardiovascular diseases in apparently healthy individuals and also predicts prognosis of AMI patients[80].

Myoglobin is a heme protein and is a non-specific cardiac biomarker which is released earlier in case of AMI and is useful in early diagnosis of AMI patients in the emergency room especially in NSTEMI patients.

To our knowledge this is one of the first studies in India utilising UWS to evaluate cardiac biomarkers in AMI patients.

In our study there was a male preponderance in group I 82.1% (n=23) compared to 17.9 % of female (n=5) which is similar to other study[57] done in AMI and non-AMI controls to evaluate CK-MB levels in UWS. This gender distribution is also similar to the demographic profile of CAD patients in Indian population published by Gupta S *et al*[113].

Our study showed an increase in percentage of STEMI patients (60.7%) compared to NSTEMI (30.9%) patients which is in concurrence with the findings of Gupta S *et al*[113] (75.4% and 24.6% respectively).

In our study the age range of patients with AMI in group I is 35-71 years and the overall mean age is 54.8 years which is in concordance with the findings published in a review by Indian council of medical research[73].

In our study 3 serial collections of UWS was done in patients admitted to the cardiac care unit, first sample at 6 -12 hours, second sample at 24- 48 hours and third sample at 48 -72 hours was collected after the onset of signs and symptoms of AMI, to investigate the rise and fall of CRP and myoglobin and to evaluate the utility of UWS as a useful diagnostic tool in early diagnosis of AMI. This serial collection of UWS is similar to other studies done in saliva and serum to evaluate cardiac biomarkers [57,104] as opposed to studies which relied on one step collection of saliva to evaluate biomarkers in patients with CAD[7, 78].

The collected UWS samples were centrifuged and the supernatant was separated and stored in aliquots at -70 degree Celsius till analysis and this is in concordance with other studies that investigated cardiac biomarkers in UWS[7,77,57].

The results of our study from analysing UWS for CRP using a high-sensitive immunoturbidimetric assay showed that hs-CRP expression is seen in all the samples collected from AMI patients (group I) and non-AMI controls (group II A + II B) in the range of 0.2 to 1.47mg/L, there was no statistically significant difference in salivary hs-CRP expression among the study groups but there is an increased expression (not statistically significant-Table 4) in the group I (AMI patients) population in all the 3 samples collected at different time period compared to non-AMI controls concurrent with the findings that CRP levels are significantly ($P<0.01$) increased in patients with AMI than normal individuals[8,78]. Among group II which is subdivided into II A- controls with periodontitis and II B - normal healthy controls without periodontitis there was no statistically significant difference in salivary hs-CRP expression and the expression levels were not higher in controls with periodontitis as compared to normal controls, this finding was different from the previous study findings that showed that hs-CRP levels are higher in individuals with periodontitis[77].

The hs-CRP levels in group I did not show any difference in saliva collected at different time periods contrary to increase seen in serum CRP levels at 36-48hours from the baseline value reported by Habib *et al*[114] and in a study similar to ours the CRP levels were found to be increased after alcohol septal ablation upto 4 fold at the 10^{th} hour post procedure from baseline value[104].

The hs-CRP levels between STEMI and NSTEMI patients in group I at the 3 time periods were not different (P=0.132,0.241and 0.379) in our study as opposed to the findings by Habib *et al*[114] in serum CRP levels which was seen to be increased in STEMI patients.

The similarity in salivary hs-CRP expression among our study groups should be further studied by comparison to serum levels. In one study[77] the salivary CRP level was found to be higher in periodontitis patient by a novel lab-on-chip using an Electronic taste chip (ETC) approach utilizing micro beads of greater surface area for more antigen/antibody interaction, in our study we used a high sensitive immunoturbidimetric assay which is used to analyse serum hs-CRP, so the use of saliva in the assay could have caused low expression of the specific epitope of the CRP antigen.

The salivary myoglobin expression in our study was evaluated using ELISA-sandwich technique which utilises 2 monoclonal antibodies to quantitatively detect myoglobin levels showed negative expression i.e. levels below the absorbance level of the spectrometer in ELISA reader (450 nm) in 60.7% of the samples in group I , in 16.6% of samples in group II A and 15.3% of samples in group II B, this negative values could be due to undetectable levels of myoglobin in saliva or due to insufficient sensitivity of the technique to identify the analyte or could be due to the matrix effect of the sample, which could lead to masking of the target protein marker by other interferences in the sample[115].

The expression of salivary myoglobin in our study showed no difference in the expression levels collected at 6-12 hours from group I when compared to group II (II

A + II B). These findings are contrary to the findings of previous studies done in serum myoglobin estimation which proved that myoglobin is the first marker to rise after MI and it peaks in 12 hours after the onset of symptoms but can be detected as early as 90 minutes and can be used to rule out or rule in AMI in emergency room[108, 109, 110].This discrepancy could be attributed to lower level of expression of myoglobin in saliva than in serum.

The expression of salivary myoglobin was significantly higher in Group II A and II B (non-AMI controls) when compared with saliva collected at 24-48hours (P= 0.009) and 48-72 hours (P=0.015) from group I (AMI patients) as opposed to a study that have shown that there is significant rise in myoglobin levels in serum as well as in UWS of AMI patients[111.] This discrepancy could be explained by the fact that there was a difference in time of collection and storage of the samples among group I and group II, while the samples from AMI patients were collected over a period of 6 months and collected from different centres in Tamil Nadu and has to be stored in the facility where we collected saliva at -20 degree Celsius before transportation to -70 degree Celsius and has undergone more than one freeze-thaw cycle (at least 2) when compared to the control samples which was collected 2 months before analysis and from a single centre and constantly stored at the same temperature and underwent only a single freeze thaw cycle before analysis. This is reflected in the study, while only 40% of the samples from group I showed positive expression, 84% of samples from group II A and 85% of samples from group II B showed positive expression for myoglobin.

The expression of myoglobin concentration in group I showed a significant increase (P=0.018) in samples collected at 6-12 hours than samples collected at 24-48

hours and 48-72 hours. This finding is in concurrence with the kinetics of myoglobin which peaks at 12 hours and falls at 36 hours [12,107].

The expression of salivary myoglobin concentration among STEMI and NSTEMI patients in group I was significantly increased (P=0.043) in the sample collected at 6-12 hours, which is similar to the findings in previous studies done to evaluate serum myoglobin for early diagnosis of AMI[108,109,110]. Moreover the myoglobin expression at 6-12 hours sample was higher in NSTEMI patients which is important as the salivary myoglobin values in absence of ECG changes can help in early diagnosis of AMI and prompt treatment.

Summary and Conclusion

- Salivary hs-CRP expression was seen in all the samples of study population
- Expression of salivary hs-CRP were higher in group I than Group II A and II B, however there was no difference when multiple comparison was done.
- Expression of salivary hs-CRP did not show any difference among group I samples collected at 6-12 hours, 24-48 hours and 48-72 hours.
- Expression of salivary hs-CRP did not show any difference between STEMI and NSTEMI patients in group I.
- Salivary myoglobin concentrations were expressed in 30.9% of the group I, 84% in group II A and 85% in group II B samples.
- Expression of salivary myoglobin concentration was higher in group II B than group II A which was in turn higher than group I.
- Expression of salivary myoglobin showed a significant increase in Group II A and II B samples when compared with 24-48 hours and 48-72 hours samples of group I (P=0.013 and P= 0.022 respectively)
- Expression of salivary myoglobin levels within group I showed a significant increase in samples collected at 6-12 hours (P=0.018) than from samples collected at 24-48 hours and 48-72 hours.
- Expression of salivary myoglobin levels in group I showed a significant difference between STEMI and NSTEMI patients in samples collected at 6-12 hours(P=0.043) the expression was higher in NSTEMI patients.

The present study shows that hs-CRP and myoglobin can be detected both qualitatively and quantitatively in saliva. While hs-CRP using Immunoturbidimetry showed consistent results, myoglobin results by ELISA were inconsistent, this inconsistency we feel was due to its sensitivity to long term storage. Based on this finding it is preferable to analyse the samples immediately or prevent repeated freeze-thaw cycle. Salivary analysis of cardiac biomarkers needs more sensitive techniques before they can be utilised as a diagnostic tool and further studies are to be done to arrive at a reference range for salivary hs-CRP and myoglobin levels.

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Annexures

ANNEXURE –I A

INSTITUTIONAL REVIEW BOARD APPROVAL

26.09.2011

From,

Institutional Review Board,

Ragas Dental College and Hospital,

Uthandi, Chennai

The dissertation topic titled "**Study of High sensitive C-reactive protein and Myoglobin in the saliva of Acute Myocardial Infarct Patients**" submitted by Dr.P.Shanmugapriya has been approved by the Institutional Review Board of Ragas Dental College and Hospital on 26th September 2011.

Dr.K.Ranganathan
Secretary,
Ragas, IRB

Dr.S.Ramachandran
Chairman,
Ragas, IRB

ANNEXURE I B

INSTITUTIONAL ETHICS COMMITTEE
THE MADRAS MEDICAL MISSION
No. 4-A, Dr. J.J. NAGAR, MOGAPPAIR, CHENNAI - 600 037, INDIA
Call : + 91 - 44 - 26561801, 26565961, 26565991 Fax : 91 - 44 - 26565859
E-mail : icvddoctors@mmm.org.in Website : http://www.mmm.org.in

To,

06th JUN2012

Dr.Shanmugapriya,
Principal Investigator,
Department of Oral and Maxillofacial pathology,
Ragas Dental College and Hospitals,
Uthandi,
Chennai.

Reference: Study of Biomarkers in the saliva of Acute Myocardial Infarct (AMI) patients.

Sub: Ethics Committee approval of study document for the above mentioned study.

Dear Dr.Shanmugapriya,

We have received from you 06 copies of each of following study related document submitted vide letter dated: 24th May 2012.

S.No	Document
1.	Protocol

At the Ethics Committee meeting held on 02nd Jun 2012, your referenced letter and the above document were examined and discussed. After due consideration, the committee has decided to approve the above-mentioned document.

The following members were present at the meeting held on 02nd Jun 2012 at 9-30 AM at Mount Tabor Lounge, Madras Medical Mission.

Sr. no.	Name of the Member	Designation and Qualification	Representation as per Schedule Y	Gender	Affiliation with the Institution
1	Dr M S Ramachandran	Senior Medical Consultant & Diabetologist, St. Isabel's Hospital, Chennai, MD, FRCP (Glasgow)	Chairman	M	N
2	Dr V M Kurian	Sr Consultant cardiovascular	Member	M	Y

		Surgeon Madras Medical Mission MS MCh DPMR	secretary		
3.	Dr.Chitrasree. V	Consultant – Laboratory Services MBBS,DCP	Basic Medical Scientist	F	Y
4.	Dr Mullasari S Ajit	Director, Cardiology MD DNB DM	Clinician	M	Y
5.	Dr. Saravanan Sundararaj	Director, ILKOT MBBS,FRCS	Clinician	M	Y
6.	Dr Philomena Mariados	Dean, Academy of Medical Sciences, PhD Madras Medical Mission	Lay Person	F	Y
7.	Fr Ninan Chacko	Chaplain, Madras Medical Mission MA,DPS	Theologian	M	Y
8.	Mr. Achuthan	B.Sc , B.L	Legal Expert	M	N

It was to be noted that neither you nor any of your proposed study team members were present during the decision-making procedures of the Ethics Committee.

Yours truly,

Signature: _____ **Date:** 6/6/12

Name: Dr V M Kurian
Title: Member Secretary

MEMBER SECRETARY
INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL MISSION
No. 4 - A, Dr. J. J. NAGAR,
MOGAPPAIR, CHENNAI - 600 037.

ANNEXURE II

INSTITUTIONAL REVIEW BOARD PROTOCOL

TITLE OF THE PROPOSED RESEARCH PROJECT

Study of High-sensitive C-reactive protein and Myoglobin in the saliva of Acute Myocardial Infarct (AMI) patients.

NAME AND DESIGNATION OF THE PRINCIPAL INVESTIGATOR

P.Shanmugapriya
2nd Year Post graduate student
Department of Oral and Maxillofacial Pathology

NAME OF THE HOD & STAFF IN CHARGE

Dr Ranganathan K. MDS, MS (Ohio), PhD. (Professor and Head)
Dr Umadevi K. MDS (Professor)
Dr Elizabeth Joshua. MDS (Associate Professor)
Dr Rooban T. MDS (Associate Professor)

Department where project is to be carried out:

Department of Oral and Maxillofacial Pathology,
Ragas Dental College,
Chennai

DURATION OF THE PROJECT: One Year

Time line: First 8 months -Sample collection
1 month -ELISA procedure
3 months -Write up and preparation for submission

Signature of principal investigator _____

Signature of Head of Department _____

Remarks of committee _____

Permission Granted YES / NO

Modifications / comments _____

RATIONALE:

For adults with chest pain, the ECG and measures of serum biomarkers are used to screen and diagnose myocardial necrosis.
These measurements require time and delays therapy and affects prognosis.
The objective of this study is to investigate the feasibility and utility of saliva as an alternative diagnostic fluid for identifying biomarkers of acute myocardial infarction.

NULL HYPOTHESIS:

Levels of hs-CRP and MYO are same in the saliva and serum of AMI patients.

AIM:

To evaluate the presence of high sensitive C-reactive protein (hs-CRP), Myoglobin(MYO) in the unstimulated whole saliva (UWS) of acute myocardial infarct patients and controls.

OBJECTIVE:

- To evaluate the presence of high sensitive C-reactive protein (hs-CRP) in the UWS of AMI patients using Immunoturbidimetric assay (IT).
- To evaluate the presence of myoglobin (MYO) in the UWS of AMI patients using Solid phase Sandwich- Enzyme linked immunosorbant assay (ELISA).
- To evaluate the presence of hs-CRP, and MYO in the UWS of controls.

MATERIALS AND METHODS:

Study groups

Group I - Unstimulated saliva from 30 acute myocardial infarct patients
Group II - Normal subjects (controls)
IIA - Unstimulated saliva from 15 controls with periodontitis
IIB - Unstimulated saliva from 15 apparently healthy controls without periodontitis

Criteria used for assessing Periodontitis:

1. Plaque scores more than 30% of sites
2. Bleeding scores more than 30% of sites
3. Probing depth greater than or equal to 3mm
4. Clinical attachment loss greater than or equal to 2mm

METHOD OF COLLECTING SALIVA:

Unstimulated saliva from AMI patients would be collected thrice, first sample within 6-12 hrs of onset of signs and symptoms of AMI, second sample after 24 hrs and before 48 hrs of onset of sign and symptoms and third sample after 48 hrs and before 72 hrs.. The subjects would be requested to spit the saliva into a sterile container to collect approximately 5 ml of saliva.

Unstimulated whole saliva would be collected from controls at a specific time, between 9 to 12 am, the subjects should be refrained from eating, drinking for at least 1hr before evaluation. The subjects would be requested to expectorate all saliva into sterile container to collect approximately 5 ml of saliva.

STORAGE:

Collected samples would be stored at -70 ^{o}C for further analysis.

PROCEDURE:

Enzyme Linked Immunosorbant Assay for Myoglobin and Immunoturbidimetric assay for hs-CRP estimation.

STATISTICS TO BE USED:

Analysis of variance (ANOVA)
Data analysis to be done using SPSS (Statistical Package of Social Science) version17

Armamentarium:

1. Oral examination devices
   Gloves, Mask, Mouth mirror, Williams probe
2. Centrifuge Tubes (1.5ml)
3. Micropipettes
4. Micropipette tips (20µl, 100µl, 200 µl,1000 µl)
5. Cooling Centrifuge
6. -70 degrees Celsius ultra low temperature storage cabinet
7. Sterile containers for saliva collection
8. Ice pack (for transfer)
9. MYOGLOBIN ELISA kit (Biocheck inc^{TM}.USA)
   Automatic washer
   ELISA reader
10. HsCRP Immunoturbidimetric kit (BioSystems S.A^{TM}, Spain)
    CS400 automatic analyser

Annexure III

Consent Form

I,…….. s/o, w/o, d/o……, aged about…..……. years, Hindu/Christian/Muslim/ …….residing at ……
…, do hereby solemnly and state as follows. I am the deponent herein; as such I am aware of the facts stated here under.

I was informed and explained about the pros and cons of the study/test in the …… language known to me.

I was also informed and explained that the results of the individual test will not be revealed to the public. I give my consent after knowing full consequences of the dissertation/thesis/study and I undertake to cooperate with the doctor for the study.

I am also aware that I am free to withdraw the consent given at any time during the study in writing.

Signature of the Patient/Attendant

The patient was explained the procedure by me and he has understood the same and signed in

(English/Tamil/Hindi/Telugu/…….) before me.

Signature of the Doctor

Annexure IV : Case History Data - Group I

SAMP .NO	AGE	GENDER	DATE	SY-ONSET	ECG	TIME-OF SAMPLE COLLECTION - Hrs			GINGI VITIS	PERIO DNT	SYS.DIS	HABITS	MEDICINES	CONTMN	Saliva-HS-CRP(mg/l)			Saliva-MYO(ng/ml)			TROP-I (ng/ml)
						A	B	C							6-12hrs	24-48hrs	48-72hrs	6-12hrs	24-48hrs	48-72hrs	(Serum-values)
MI 001	42	M	9.6.12	6am	STEMI	12	28	50	YES	YES	HTN	Alcohol	Aspirin,clop,pan	A-BLOOD	0.73	0.53	0.53	3.596	0.558	0.533	Neg,.021
MI 003	55	M	16.6.12	4:30am	STEMI	12	27	54	YES	YES	Type 2 DM(new)	NIL	Ecos,Clop,Atorva,Amit	B-<1.5ml	1.46	0.57	0.59	0.978	1.011	0.643	1.02(16.6.12)/80.0-17.6.12
MI 005	65	M	21.6.12	9pm(20.6)	STEMI	11	35	59	NO	NO	Type 2 DM,HTN	NIL	Ecos,Clop,Storvas,Alprax,Amaryl		0.68	0.68	0.52	0.738	0.952	0.147	1.34 & 48.5-(21.6.12)
MI 007	54	M	26.6.12	6pm(25.6)	STEMI	13	38	62	YES	YES	NIL	NIL	Ecos,Clop,Storvas,Amit.	A-Bld	0.76	1.47	0.59	1.375	-0.86	0.584	0.419(25.6.12)/80.0(26.6.12)
MI 009	46	M	4.7.12	1:45am	STEMI	6	30	54	NO	NO	NIL	NIL	Ecos,Clop,Storvas,Pan		0.76	0.38	0.69	0.777	0.516	1.742	7.24(4.7.12)/11.6(5.7.12)
MI 010	67	M	8.7.12	10:30am	STEMI	10	30	54	YES	YES	Type 2 DM(new)	NIL	Ecos,Clop,Storvas,Pan		0.63	0.2	0.76	-3.33	-9.69	-16	0.420(8.7.12)/40.0(9.7.12)
MI 011	39	M	9.7.12	1:45pm(8.7	STEMI	16	30	54	YES	NO	Type 2DM(new)	Smoking	Ecos,Clop,Storvas,Pan		0.76	0.66	0.5	0.243	-0.13	-0.17	>80(8.7.12)/54.4(9.7.12)
MI 016	50	M	16.7.12	6am	STEMI	5	24	48	YES	NO	NIL	NIL	Aspirin,clop,pan		0.6	0.38	0.71	0.571	0.371	-0.93	NA
MI 017	60	M	18.7.12	8am	STEMI	9	26	50	NO	NO	NIL	NIL	Ecos,Clop,Storvas,Pan		0.44	0.46	1.28	-1.35	0.316	0.758	Neg<0.012
MI 021	64	M	8.9.12	10pm	STEMI	10	34	58	YES	YES	NIL	NIL	Ecos,Clop,Cardivas		0.71	0.44	0.5	8.828	4.493	1.645	>80.0(7.9.12)
MI 022	56	M	13.9.12	8pm(12.9)	STEMI	12	24	NA	YES	YES	Type 2 DM,HTN	NIL	Ecos,Clop,Storvas,Pan	C-NA	0.46	0.5	NA	-1.71	-5.48	NA	NA
MI 026	71	M	15.8.12	7am	STEMI	9	28	52	YES	YES	NIL	NIL	Aspirin,Clop,Isordil,Raciper		0.72	0.63	0.68	10.23	3.998	1.444	NA
MI 027	52	M	21.9.12	9pm	STEMI	13	42	61	YES	NO	NIL	NIL	Ecos,Clop,Atorva,Pan		0.84	0.73	0.44	5.771	-4.16	-3.95	NA
MI 028	71	M	16.8.12	9pm	STEMI	12	36	60	YES	YES	Type 2 DM	NIL	Aspirin,Clop,I.Emeset	A-<1ml	NA	0.62	0.3	8.29	1.435	-0.21	NA
MI 030	61	M	24.9.12	9:30pm	STEMI	12	36	NA	YES	NO	HTN	NIL	Ecos,Clop,Storvas,Pan,Ansit	C-NA	0.78	0.62	NA	-2.18	-2.6	NA	NA
MI 033	59	M	4.10.12	11am(3.10)	STEMI	20	32	NA	YES	YES	Type 2 DM,HTN	NIL	Ecos,Clop,Storvas	C-NA	0.4	0.44	NA	1.686	1.029	NA	>6.1(4.10.12)
MI 035	58	M	8.11.12	12pm	STEMI	7	22	48	YES	YES	Type 2 DM	Smoking	Disp,Losoar,Isordil		0.88	0.46	0.65	-5.74	-15.7	-11.8	NA
MI 002	65	M	13.6.12	8pm(12.6)	NSTEMI	17	35	59	YES	YES	HTN	NIL	Ecos,,Storvas,Pan,Ansit		0.73	0.72	0.59	0.951	-0.08	0.609	0.238(12.6.12)0.755(15.6.12)
MI 004	62	F	20.6.12	12pm	NSTEMI	6	25	52	YES	YES	Type 2 DM	NIL	Ecos,Clop,Storvas,Pan,Ansit		0.59	0.57	0.73	1.775	2.174	1.297	Neg 0.078
MI 006	52	M	14.6.12	6am	NSTEMI	5	26	47	YES	NO	Type 2 DM	smoking			0.5	0.72	0.59	2.053	0.867	-0.32	NA
MI 008	47	F	23.6.12	3am	NSTEMI	4	24	43	NO	NO	HTN	NIL	Aspirin,clop,Isordil		0.65	1.45	0.6	9.346	0.154	-7.18	NA
MI 018	42	F	24.7.12	10am	NSTEMI	4	23	45	YES	NO	Type 2 DM	NIL	Aspirin,clop,Isordil		0.48	0.71	0.65	1.005	1.57	2.332	NA
MI 019	45	M	24.7.12	12:30pm	NSTEMI	3	24	49	YES	YES	Type 2 DM	NIL	Aspirin,clop,Isordil		0.84	0.69	0.71	1.588	-7.24	-0.22	NA
MI 020	58	F	24.7.12	6:30am	NSTEMI	5	26	48	YES	YES	HTN	NIL	Aspirin,clop,Isordil	A-Sput	0.52	0.4	0.59	4.062	-4.04	-2.42	NA
MI 024	51	M	2.8.12	6:30am	NSTEMI	5	24	52	YES	NO	Type 2 DM	NIL	Aspirin,clop,Isordil		0.84	0.78	0.66	8.407	8.755	4.204	NA
MI 025	46	M	2.8.12	8am	NSTEMI	4	26	49	YES	YES	HTN	NIL	Aspirin,clop,Isordil		0.57	0.57	0.72	5.057	-0.91	-0.86	NA
MI 029	62	M	14.8.12	1pm	NSTEMI	7	31	54	YES	YES	Type 2 DM,HTN	Smoking	Ecos,Clop,Storvas,Pan,Ansit	A-Bld	0.15	0.79	1.28	3.474	2.79	1.666	>1.76(15.8.12)
MI 031	35	F	3.10.12	5:30am	NSTEMI	11	28	NA	YES	NO	Type 2 DM,HTN	NIL	Ecos,Clop,Pan,Ansit	C-NA	0.62	0.33	NA	-1.45	-3.29	NA	780.0(3.10.12)
MI 023	67	M	15.9.12	8pm(14.9)	STEMI	12	NA	NA	YES	YES	Type 2 DM	NIL	Ecos,Clop,Storvas,Pan,Ansit	B&C NA							>80.0(14.9.12)
MI 012	62	F	10.7.12	5:30pm(9.7)	NSTEMI	13	NA	NA	YES	YES	Type 2 DM,HTN		Ecos,Clop,Storvas,Pan	B&C NA							0.393(9.7.12)

12 AND 23 WERE NOT USED AS SAMPLES AT 24 -48 HOURS (B) AND 48-72 HOURS (C) COULD NOT BE COLLECTED.

NA Not available

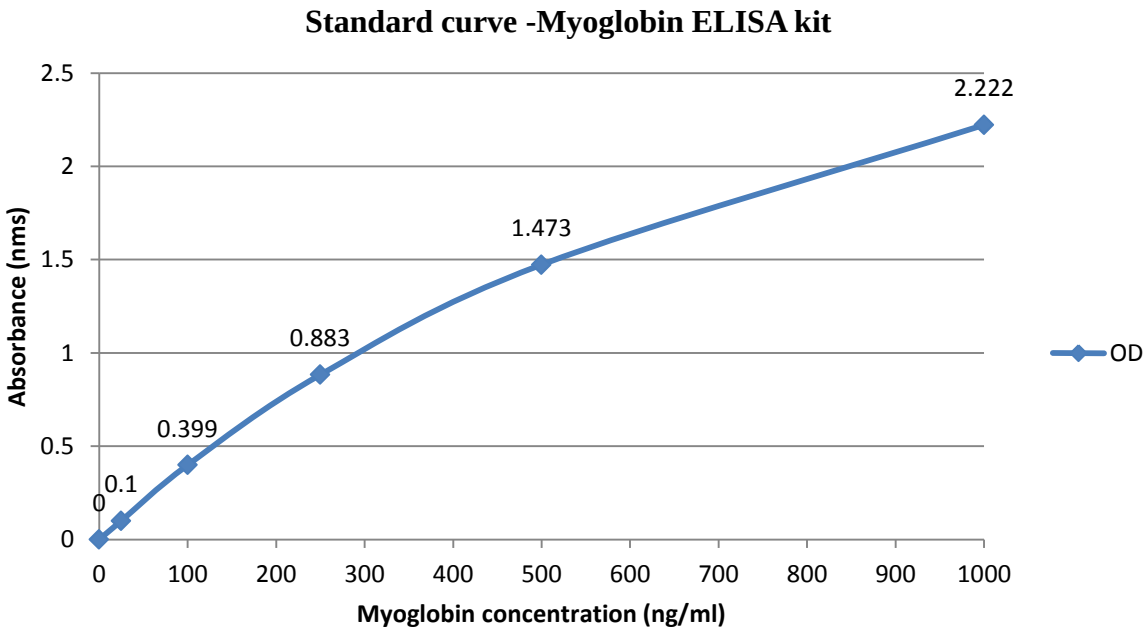
Annexure IV- Case History Data- Group II (IIA + II B)

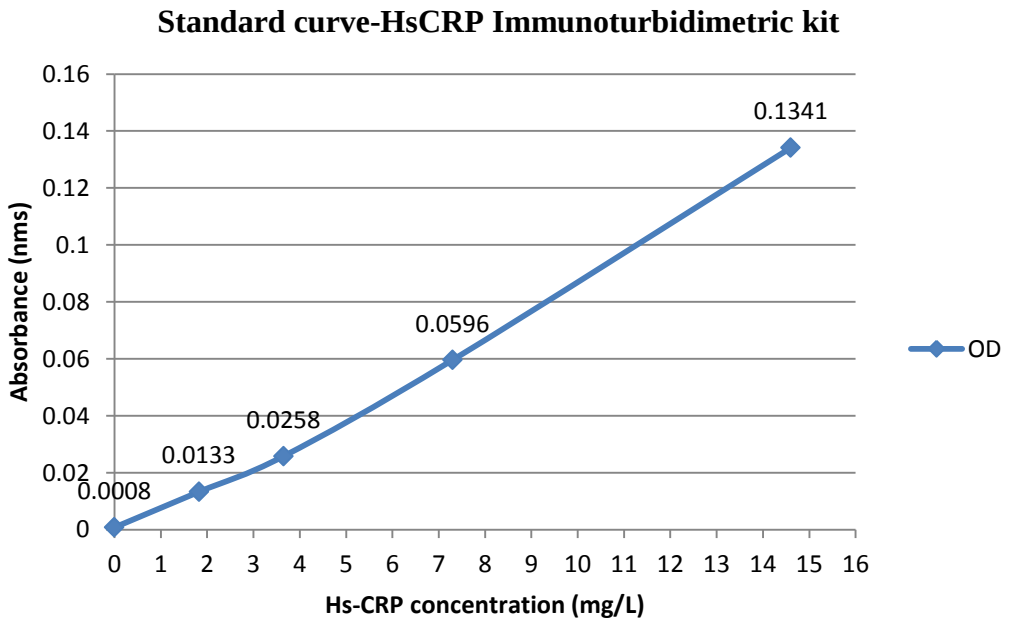
CASE NO	SAMPLE NO	DATE	AGE	GENDER	GINGIVITIS	PERIODONTITIS	SYST.DISEASES	HABITS	HsCRP (mg/L)	MYO (ng/ml)
1	CP 05	11.9.12	52	M	YES	Gen.Chronic	Type 2 DM	Smoking		
2	CP 06	13.9.12	35	M	YES	Localised	NIL	NIL	0.69	4.264
3	CP 07	15.9.12	42	M	YES	Localised	NIL	NIL	0.62	0.208
4	CP 08	20.9.12	63	M	YES	Gen.Chronic	Type 2 DM	NIL	0.48	1.953
5	CP 09	22.9.12	45	M	YES	Gen.Chronic	NIL	Smoking	0.68	10.23
6	CP 10	25.9.12	52	M	YES	Gen.Chronic	NIL	NIL	0.24	-1.09
7	CP 13	25.9.12	63	M	YES	Gen.Chronic	Type 2 DM	NIL	0.44	4.225
8	CP 01	6.8.12	40	F	YES	Localised, severe	NIL	NIL	0.55	0.582
9	CP 02	11.8.12	38	F	YES	Gen.Chronic	NIL	NIL	0.68	0.703
10	CP 03	16.8.12	55	F	YES	Gen.Chronic	HTN	NIL	0.59	0.774
11	CP 04	17.8.12	50	F	YES	Gen.Chronic	NIL	NIL	3.18	272.4
12	CP 11	20.8.12	60	F	YES	Gen.Chronic	Type 2 DM	NIL	0.57	22.24
13	CP 12	20.8.12	41	F	YES	Localised.	NIL	NIL	0.5	-5.58
14	CP 14	2.9.12	59	F	YES	Gen.Chronic	NIL	NIL	0.63	2.139
15	CP 15	6.9.12	35	F	YES	Localised	NIL	NIL	0.63	1.693
16	CN 01	16.8.12	40	M	NO	NO	NIL	NIL	0.52	5.344
17	CN 03	16.8.12	55	M	NO	NO	NIL	NIL	0.71	10.74
18	CN 08	20.8.12	30	M	NO	NO	NIL	NIL		
19	CN 09	10.9.12	31	M	NO	NO	NIL	NIL		
20	CN 12	12.9.12	43	M	NO	NO	NIL	NIL	0.57	-2.022
21	CN 13	14.9.12	65	M	NO	NO	NIL	NIL	0.79	2.098
22	CN 02	18.8.12	54	F	NO	NO	NIL	NIL	0.55	1.034
23	CN 04	19.8.12	55	F	NO	NO	NIL	NIL	0.87	0.682
24	CN 05	19.8.12	45	F	NO	NO	NIL	NIL	0.55	3.542
25	CN 06	20.8.12	35	F	NO	NO	NIL	NIL	0.59	11.63
26	CN 07	20.8.12	40	F	NO	NO	NIL	NIL	0.62	16.85
27	CN 14	16.9.12	40	F	NO	NO	NIL	NIL	0.72	8.259
28	CN 16	22.8.12	40	F	NO	NO	NIL	NIL	0.69	3.894
29	CN 17	8.11.12	34	F	NO	NO	NIL	NIL	0.62	-5.79
30	CN 18	8.11.12	34	F	NO	NO	NIL	NIL	0.35	0.971

CASE NO 1, 18 AND 19 SAMPLES WERE NOT INCLUDED BECAUSE OF SAMPLE CONTAMINATION

CASE NO 11 AND 12 SAMPLES WERE NOT INCLUDED IN RESULTS BECAUSE OF ABNORMAL VALUES

ANNEXURE V


Standard curve -Myoglobin ELISA kit
Absorbance (nms)
2.5
2
1.5
1
0.5
0
0
100
200
300
400
500
600
700
800
900
1000
Myoglobin concentration (ng/ml)
2.222
1.473
0.883
0.399
0.1
0
OD


Standard curve-HsCRP Immunoturbidimetric kit
Absorbance (nms)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Hs-CRP concentration (mg/L)
0.1341
0.0596
0.0258
0.0133
0.0008
OD